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14. ABSTRACT

In the award (W81XWH-08-1-0627), the stated goals were to study the zinc transporters that may be disparately expressed in the prostate tissues of African American (AA) males as compared to European American (EA) males. This may lead to the identification of potential molecular targets for nutritional, environmental or life style factors for their potential relationship to the incidence or progression of prostate cancer in various racial groups.

The following tasks were carried out in a synergistic fashion between the laboratories of Dr. Bagasra (Claflin University) and Dr. Balla (University of Illinois, Chicago): Task 1: We measured the differential expression of four zinc transporters- *hZIP1, hZIP2,* and *hZIP3*–genes by RT-*in situ* PCR in the tissue arrays (TMA) prepared from various racial groups. We found significant decrease in hZIP1 in AA when compared to EA. No difference was found in the other two transporters (*hZIP4* was not expressed in the prostates of either AA or EA). Task 2: We determined the differential levels of the amount and location of intracellular zinc in various areas of the prostate gland as well in cells in different histological grades of cancer and in healthy prostate tissues by utilizing fluorescent zinc indicators and confocal microscopy both in AA and EA subjects. Task 3, immunohistochemical expression of these genes was not approved for funding. Tasks were accomplished, as written below.

15. SUBJECT TERMS
Prostate Cancer, zinc, zinc transporters, African-American, European-American, microRNA, health disparity

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Differential Expression of Zinc Transporters in Prostate Epithelia of Racial Groups

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Introduction

Prostate cancer (PC) is the development of a malignant tumor within the prostate gland, originating mainly in the peripheral zone, where the majority of zinc (Zn) accumulating cells are located. The underlying basis of our study focuses on the essential trace element Zn which is needed in relatively high amounts for reproductive processes. According to *Cancer Facts 2012,* men of African descent have the highest rates of PC incidence in the world. Currently, the reason for this racial disparity is not understood, but like similar diseases, may develop due to the interaction of diverse risk factors including age, race, genetics, socioeconomic and lifestyle choices, broadly defined to include geographical and nutritional factors. Previous studies we have investigated the accumulation of Zn within the prostate gland of African American men (AA) and European American men (EA), which show that AA lose zinc levels in the glandular epithelia at an earlier age and have a relatively lower accumulation of Zn within the gland. Our study aims to compare the expression of a human Zn transporting mRNAs; *hZIP1*, *hZIP2*, *hHZIP3*, and *hZIP4*, which were proposed as gene regulators in the prostate cancer development and particularly *hZIP1* as a tumor suppressor gene and appears to play a role in the molecular pathogenesis of PC. We theorize that there are significant differences in the expression of *hZIP1* in the glandular regions of the prostate between AA and EA by RT-in situ PCR. In order to quantify the level of hZIP1 expression in the glands, we analyzed 300 prostate cancer cases, 150 from AA and 150 from EA. We report here that the relative *hZIP1* mRNA expression were significantly downregulated in AA as compared to EA ($(p<0.001)$ (see detail below). This is the largest sample data ever used to date to determine the relative differences of *hZIP1* in prostate tissues, and confirms our preliminary data. Our results suggest that AA might have evolutionarily down-regulation of the *hZIP1* transporter due to high levels of Zn in the African continent. In addition, we have carried out hZIP2-hZIP4 expressions in the TMA and our preliminary analyses suggest that hZIP2 and hHZIP3 are also downregulated in the adenocarcinoma tissues of both AA and EA but there were no significant differences between the two ethnic groups. There was no amplification of hZIP4 in any of the TMA we analyzed and we believe that the expression of this zinc transporter in the prostate is absent.

In the award (W81XWH-08-1-0626), our goals are to identify an explanation for differences in prostate cancer incidence and outcome of African American (AA) males at the molecular level. The main question that we are attempting to answer is "Are there any consistent differences in the expression of significant genes or proteins in the prostate cancers taken from AAs *versus* those from European Americans (EAs)?" Because there is a well-documented depletion in zinc levels with neoplastic conversion of normal prostate cells, the study had initially focused on the expression of genes known as zinc transporters. The hypothesis is that the expression levels of one or more of zinc transporter genes (*hZIPs*), as measured by mRNA expression may be differentially expressed in AAs and EAs. A study of the genes and proteins which influence the expression of any gene confirmed to be disparately expressed might lead to the identification of one or more environmental or living pattern factors worthy of epidemiological research for its potential relationship to the incidence or progression of prostate cancer in AAs, EAs or other ethnic groups.

For this purpose, we study the zinc transporters that may be differentially expressed in the prostate glandular tissues of AA males as compared to EA males. This might lead to the identification of the potential molecular targets for nutritional, environmental or life style factors.

The following tasks are to be carried out in a synergistic fashion between the laboratories of Dr. Bagasra (Claflin University) and Dr. Balla (University of Illinois, Chicago). The prostate tissues and pre-made tissue microarrays (TMAs) were obtained from the NCI -initiated "Cooperative Prostate Cancer Tissue Resource (CPCTR)" by Dr. Balla, one of the P.I.s of the present proposal and of the CPCTR. The main tissue microarray that is being used is composed of age-matched and the Gleason score-matched prostate tissues with representative tissues from 150 African American males and 150 European American males. Another type of TMA, based on long term clinical follow up was used to address the question of whether *hZIP* gene and protein expression is associated with poor outcome.

Task 1: To measure the differential expression of four main zinc transporters- *hZIP1, hZIP2, hHZIP3 and hZIP4* –genes, we will perform RT-*in situ* PCR in the tissue arrays (TMAs) prepared from various racial groups which are age, TNM stage, and Gleason Score-matched in order to determine any potential differences in the gene expression at the mRNA level between the racial groups and in normal *vs* neoplastic clusters of cells.

Task 2: To determine the differential levels of the amount and location of intracellular zinc in various areas of the prostate gland as well as in cells in different histological grades of cancer and in healthy prostate tissues by utilizing fluorescent zinc indicators and other methods.

~~**Task 3:** To develop antibodies against the four zinc transporters, we will carry out an extensive quality control protocol for these antibodies. We will perform immunohistochemical analyses of TMAs and determine the degree of each of the four *hZIP* protein expressions by semi-quantitative image analyses~~ (due to concerns that we would not be able to find suitable antibodies for this task, it was removed by the DOD following recommendations of the review panel).

BODY

"Task 1: To measure the differential expression of four main zinc transporters- *hZIP1, hZIP2, hHZIP3 and hZIP4* –genes, we will perform RT-*in situ* PCR in the tissue arrays (TMAs) prepared from various racial groups which are age, TNM stage, and Gleason Score-matched in order to determine any potential differences in the gene expression at the mRNA level between the racial groups and in normal *vs.* neoplastic clusters of cells".

Results:

The Tissue Microarrays: The Tissue Microarray (TMAs) were obtained from the NIH Cooperative Prostate Cancer Tissue Resource (CPCTR) through Dr. Andre Balla-the PI at UIC-Chicago-and each slide consisted of 150 specimens from African American (AA) and 150 from European American (EA) men (shown as pink color). We obtained four sets of ethnicity TMA slides, each set for hZIP1, hZIP2, hHZIP3 and hZIP4 determinations by the RT-in situ PCR. The TMAs also contained specimens from benign prostatic hyperplasia (BPH) (yellow) and normal prostate tissues (green). The Slides were arranged as shown below.

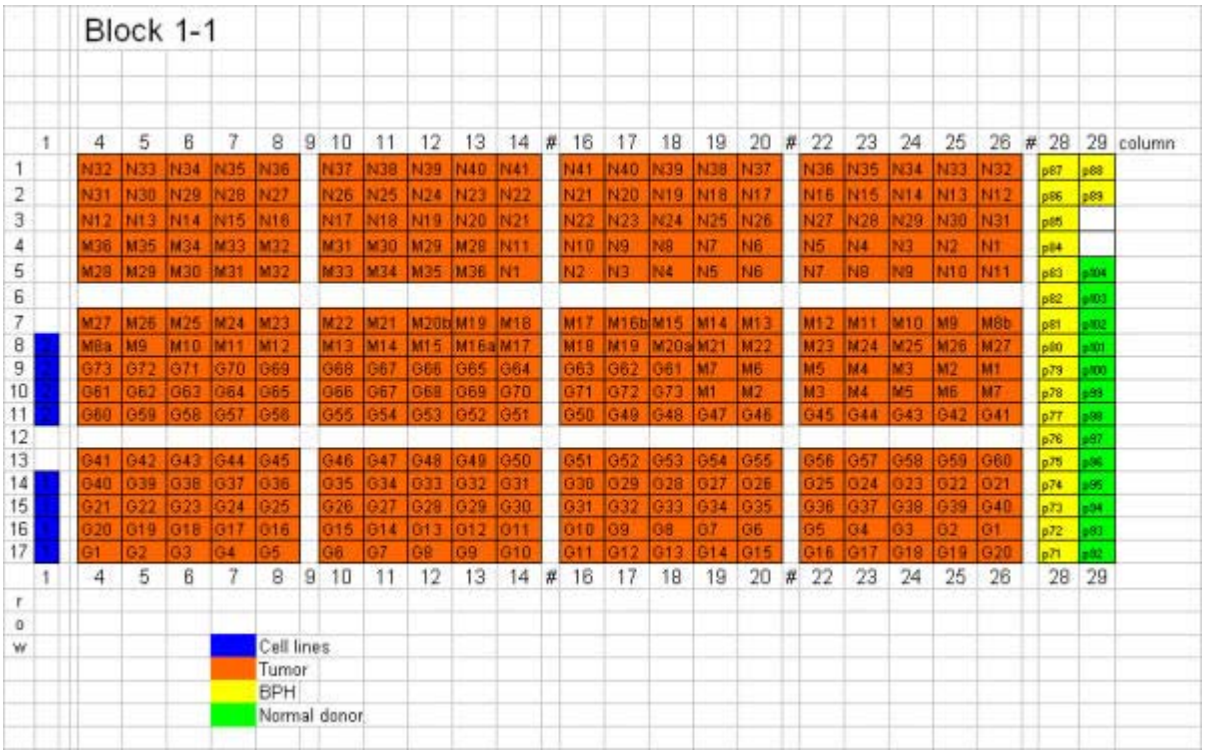


Figure 1: **Core Array Mapping Image.** Schematic image showing the array locations of cores listed in the TMA document. (ref: Berman *et al. BMC Bioinformatics* 2004; 5:19 doi:10.1186/1471-2105-5-19)

We carried out the reverse transcription based in situ PCR (RT-ISPCR) for each of the zinc transporters (i.e., *hZIP1-hZIP4*). The relative expression levels of *hZIP1* were carried out by RT-*in situ* PCR. The following primer pairs were used for amplification: sense 5'-TCAGAGCCTCCAGTGCCTGT-3' and antisense 5'-TTGTCTCCTGGACCTGCTGC-3' for hZIP1 that gave a 189-bp product. To form cDNA copy of the template and to amplify, we used one-step in situ RT-PCR enzyme – rTth enzyme, which has both the RT and polymerase function. Hybridizations were performed with 41 bp FITC (fluorescein isothiocynate) oligonucleotide probe for hZIP1 (oligonucleotide: 5'-FITC-CCTGGTGCCCATCTGTGTGCTGCGC-CGGCCAGGAGCTAAC-3'). First we provide representative *hZIP1* expressions in of TMA slides that contained adenocarcinoma, BPH and normal prostate cores. This report establishes for the first time with direct *in situ* measurements of relative *hZIP1* mRNA expressions levels are significantly decreased in prostatic adenocarcinoma as compared with normal prostate glandular epithelium or BPH. These changes occur in early stage malignancy and persist in the progression of malignancy. In addition, zinc treatment of malignant PC3 cells exhibited cytotoxic effects. These results suggest the possibility that zinc might be a tumor suppressor of prostate adenocarcinoma; and provide new insight into important factors and events in prostate carcinogenesis, which can lead to the development of early biomarkers and new efficacious therapeutic agents.

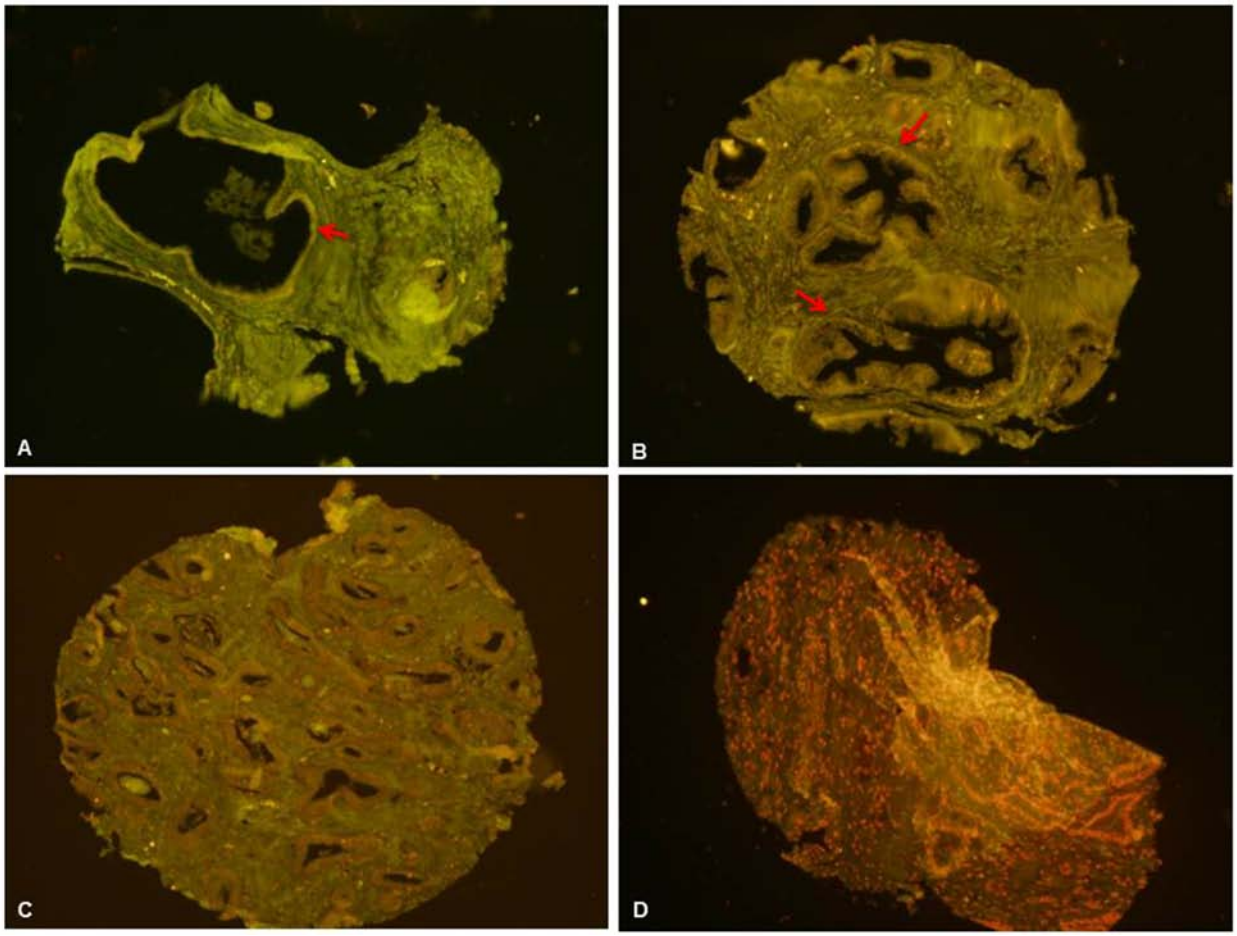


Figure 2A shows the high expression of *hZIP1 i*n a normal prostate normal prostate gland as indicated by the FITC conjugated green probe. Both stromal and the glandular areas are strongly positive (green fluorescence) in normal prostate (Fig2A) as well as in BPH (Fig 2B) tissues. However, *hZIP1* expressions were very low in the glandular areas in the malignant epithelia of EA (Fig2C) and AA (Fig2D). This is consistent with the loss of zinc that characterizes prostate cancer (2-8). (magnification 10x)

hZIP Expressions in adenocarcinoma.

hZIP transporter abundance and expression in normal prostate, BPH and adenocarcinoma.

To gain initial insight into the possible cause of the loss of zinc in adenocarcinoma, the relative abundance of hZIP1 was determined. As shown in Figure 3, representative results obtained with *hZIP1* RTISPCR staining of cores in the same TMAs employed for above analysis with approximately duplicate tissue cores (histospots) from approximately 150 AA and 150 EA subjects. *hZIP1* transporter is abundant in normal prostatic stroma as well as in the glandular epithelia loss of hZIP1 is likely to be involved in the decrease in zinc in adenocarcinoma.

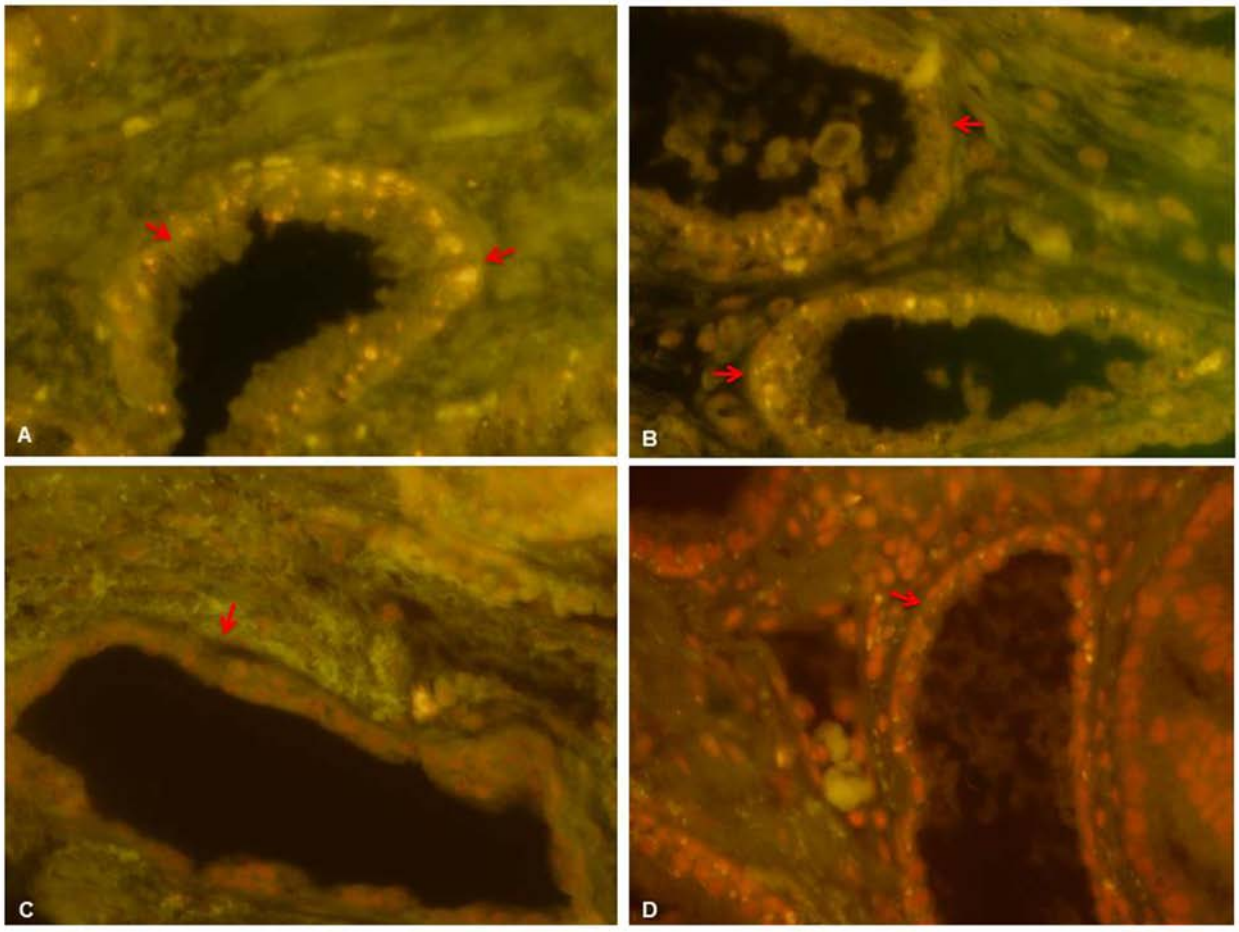


Figure 3: *hZIP*1 RTISPCR of tissue array slides and tissue sections containing normal prostate, BPH and adenocarcinoma cores. The same tissue array series as employed in Figure 2. (3A) shows *hZIP1* mRNA expression for normal at 40x magnification. (3B) BPH at 40x magnification and Figure 3C-D two adenocarcinomas at 40x magnifications. (3C is from an EA man and 3D from an age-matched and Gleason score matched AA man). We determined if the downregulation of *hZIP1* gene expression was responsible for loss of transporter in adenocarcinoma. Figure 3 clearly shows the expression of *hZIP1* in normal glandular epithelium; and the loss of expression in adenocarcinoma; which correlates with the change in *hZIP1* transporter abundance. Bright Yellowish-green = high expression of hZIP1. Red =low or absent expression of hZIP1. Of note**,** *hZIP1* shows that this transporter exist in normal glandular epithelium; and is markedly decreased in adenocarcinoma.

Ethnic Differences in the Malignant Epithelia of African American Men Vs European American Men:

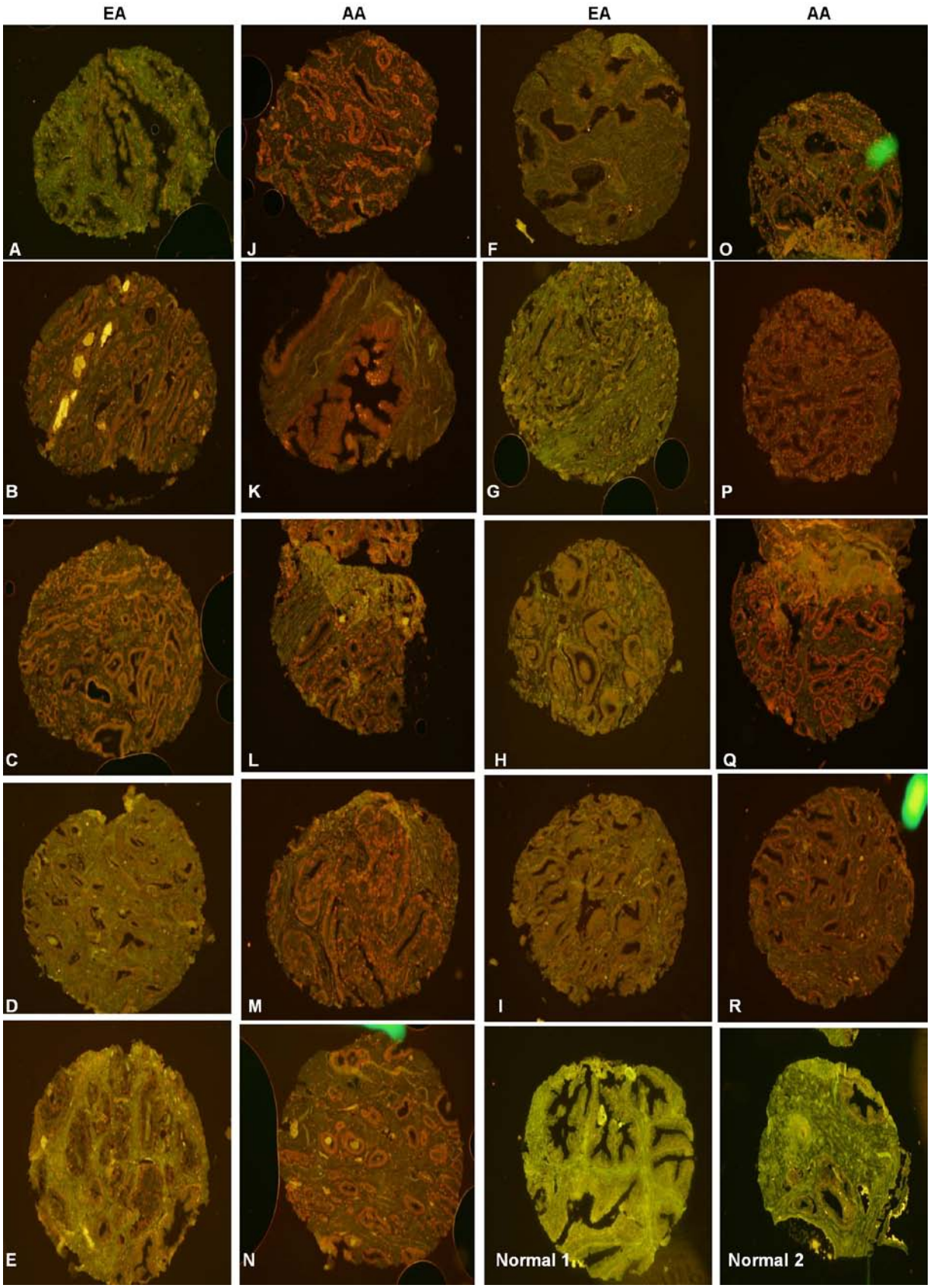
After analyses of hZIP1 expression levels in normal *Vs.* adenocarcinomas levels and establishing the differential expressions of *hZIP1* in glandular *Vs.* stromal cell types, we next examined the differential hZIP1 expressions in the two ethnic groups-EA *Vs* AA. In our previous studies we have shown by real time PCR and RT-ISPCR that hZIP1 expressions were significantly downregulated in AA as compared to EA. However, our analyses were carried out in very small group of specimens. Here we have utilized TMAs from 150 AA and 150 EA. As shown in Table 1 below, the RT-ISPCR data confirmed our previous data in the large number of cases of the ethnicity TMA that hZIP1 was significantly downregulated in AA Vs EA.

Table 1
Analyses of Tissue Micro Array (TMA)
Differential Expression of hZ1P1 mRNA in the Prostate Tissue from European American Men (EA) verses African American Men (AA)

No. of positive EA	No. of negative EA	P value	No. of positive AA	No. of negative AA	P value
127	5	<0.001	25	110	<0.001

As shown in the Table 1 above that 127/132 core biopsies from adenocarcinoma from EA (Gleason3+3) showed relatively positive *hZIP1* mRNA expressions as measured by presence of green/yellow staining, whereas only 5/132 were negative. In contract to EA the hZIP1 mRNA expressions in AA were negative in the majority of the core biopsies matching the same Gleason score. Therefore, 110/135 were negative whereas, 25/135 were determined to be positive. In each ethnic group there were some biopsies which were either deemed lost during the RTISPCR process or were unreadable. The relative degrees of expressions of hZIP1 between EA and AA were highly significant ($p<0.001$) by paired t-test. Figures 4 and 5 below show the representative figures from EA *Vs.* AA at 10x magnification and 40x magnification, respectively.

Figure 4. Representative pictures of *In situ* detection of *hZIP1* mRNA expression levels in the TMA core biopsies from European American men (A-I: columns 1 and 3) *Vs* African American men (J-R; columns 1 and 3) are shown. The abnormal glandular epithelia, exhibits a significant down-regulation of *hZIP1* mRNA as compared to the surrounding stromal areas showing a relatively higher degree of *hZIP1* expression. This figure shows the relative degrees of expressions of *hZIP*1 as determined by in situ RT-PCR/hybridization method. As one notes that in all specimens from malignant tissues (A-R), the malignant glandular epithelial areas of the prostate show significant downregulation of *hZIP1* as compared to the normal control (the bottom right figures) shows the representative figures from normal prostate biopsies. One notes significant down-regulations of *hZIP1* in AA as compared to paired EA core biopsies (final magnification 10x)


EA
AA
EA
AA
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Normal 1
Normal 2

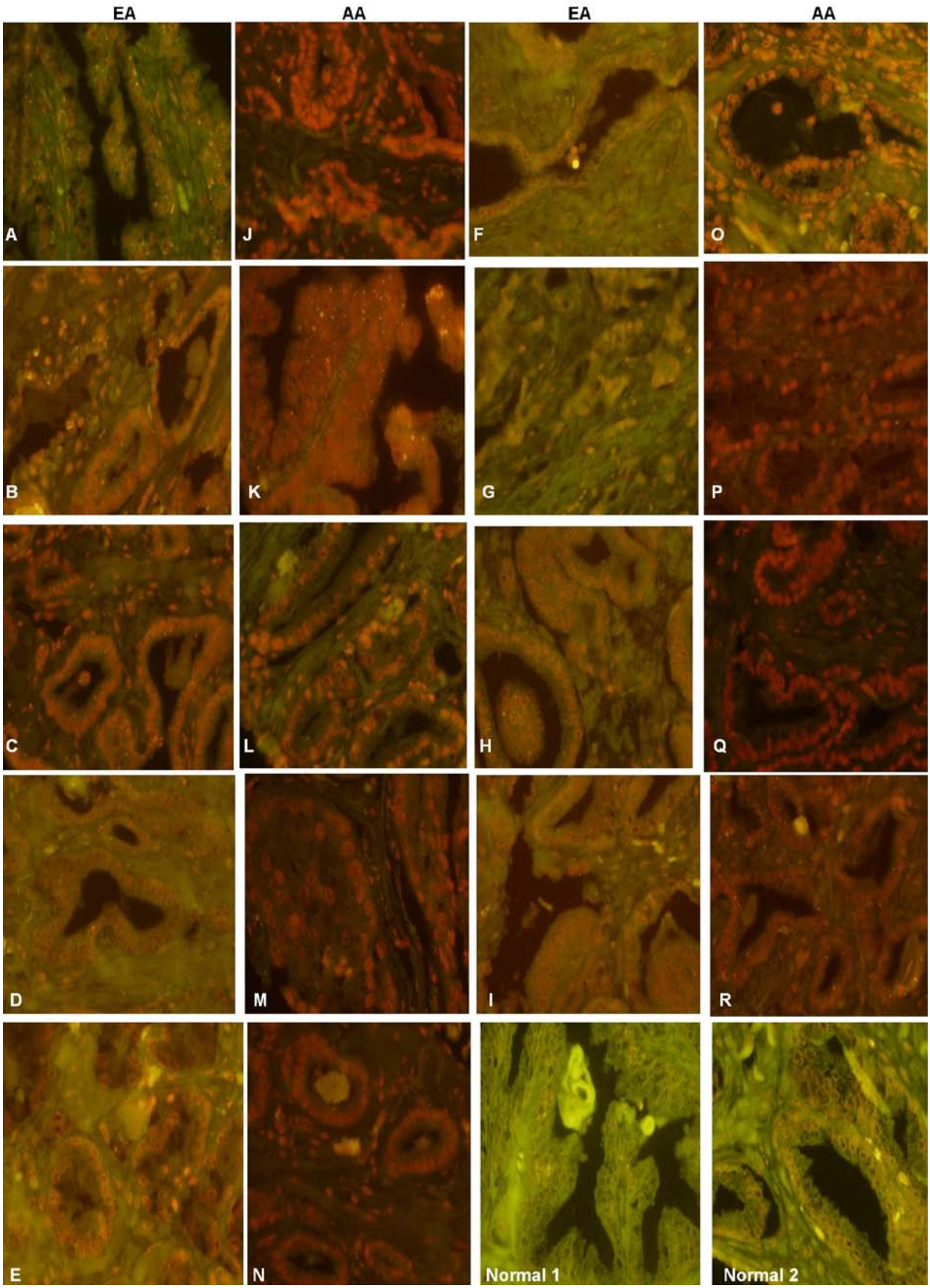
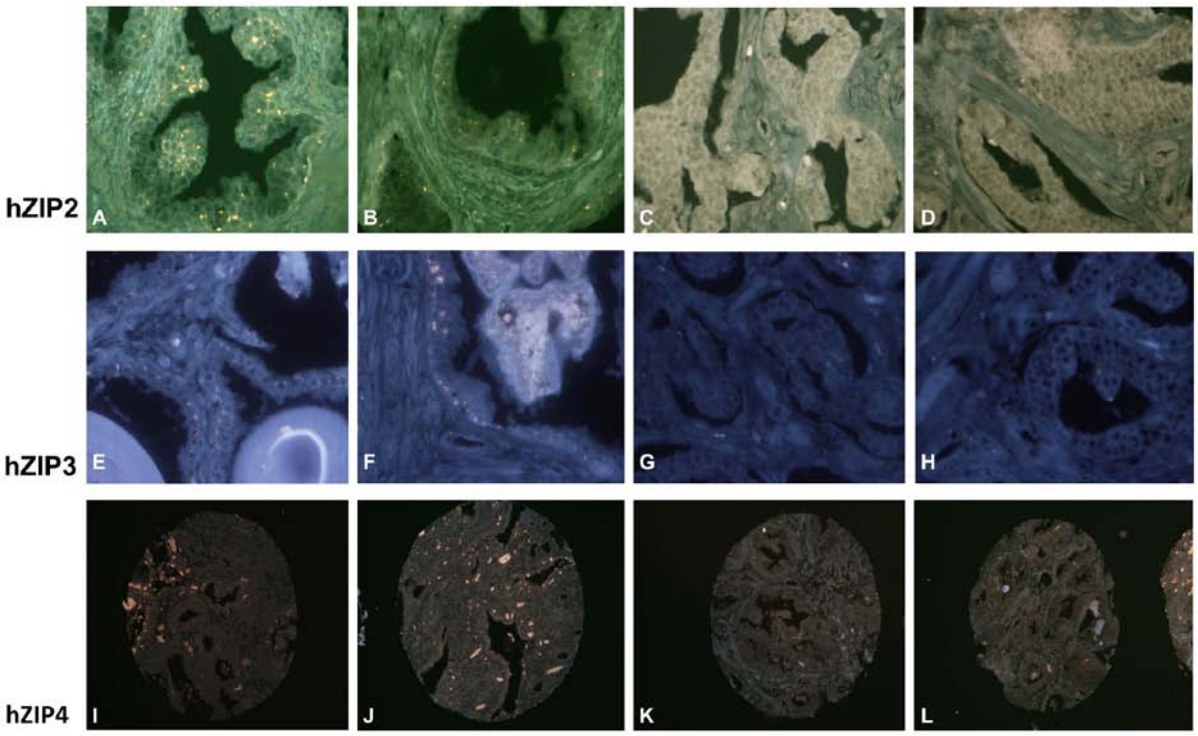


Figure 5: Representative pictures of *In situ* detection of *hZIP1* mRNA expression levels in the TMA core biopsies from European American men (A-I: Columns 1 and 3) *Vs.* African American men (J-R; Columns 2 and 4) at high magnifications (40x). Relatively high levels of *hZIP1* expressions in normal appearing glands can be

seen as a yellowish/green color on the bottom of the figures, whereas low or absent expression can be seen as red colors in malignant glands adenocarcinomas, particularly in the epithelia (EA, columns 1 and 3) of the slide and AA (columns 2and 4) of the slide. We noticed that the greenish/yellowish colors in epithelia were absent or very low in the AA core biopsies as compared to the EA specimens, suggesting relatively lower expressions of *hZIP1* in AA men. Of note, in both ethnic groups there is higher expression of *hZIP1* in the stroma than in the glandular area, but the expressions were significantly lower in the epithelia of AA as compared to EA.

***hZIP2, hHZIP3* and *hZIP4* transporter abundance and expression in normal prostate and adenocarcinoma TMA of African Americans and European Americans prostate tissues**.

To gain initial insight into the possible cause of the loss of zinc in adenocarcinoma, the relative abundance of three other cellular zinc uptake transporters; *hZIP2, hHZIP3* and *hZIP4*, were determined by RT- in situ PCR in the TMAs. **Figure 6** shows representative results obtained with *hZIP2-hZIP4* staining of cores in the same sets of the TMAs employed for *hZIP1*analyses. (**Top Panel: Cy5 probes with greenish color**). Representative pictures of are shown for *hZIP2* expressions. *hZIP2* expression was relatively higher in the normal (6A) and BPH (6B) biopsies than in and adenocarcinoma glands. Both EA (6C) and AA (6D) prostate cores appear to exhibit a downregulation of *hZIP2* in the malignant areas. However, there was no significant difference in the relative degree of expressions of this particular zinc transporter between the EA and AA tissues. (**Middle Panel: Blue color probes**). *hHZIP3* transporter was abundant in normal prostatic glandular epithelia (6E) and BPH (6F), In adenocarcinoma from EA (6G), and AA (6H) both *hHZIP3* transporter expression was down-regulated. However, again we did not observe any significant differences between EA and AA. **(Bottom Panel: Greyish color probe).** *hZIP4* expression was also evaluated but we were unable to observe any amplification of this zinc transporter in the TMAs and we conclude that hZIP4 does not play any significant role in zinc uptake in prostate tissues. **Figure 6I-L** shown representative pictures from normal, BPH, EA and AA core biopsies, respectively.



Discussion

Worldwide, there are more than 10 million new cancer cases each year, and cancer is the cause of approximately 12% of all deaths. Among all cancers, PC is the second leading cause of male

cancer- related deaths. Over 200,000 males were identified with PC in 2003 and as a result 30,000 died. In 2010 more than 186,000 US men will be diagnosed with PC and over 30,000 may die. Despite the extensive clinical and experimental studies over the recent decades, the pathogenesis of PC remains unanswered. The interaction of genetics and the environment and its influence on the molecular mechanisms responsible in the development and progression of malignant prostate cells are largely unknown. There is a great need to explore the role of differential gene expression that leads to altered cellular metabolism as an essential factor in prostate malignancy. The combination of genetic/molecular/ environmental factors and their relationships are required to identify the critical events in the prostate malignancy process. Such studies are proving to be very useful in the understanding of the molecular pathogenesis of prostate cancer.

Zinc and Zn transporters play an important role in the molecular pathogenesis of PC. PC afflicts one out of nine men over the age of 65 years. Prostatic intraepithelial neoplasia (PINs) is relatively common and occurs early in life. However, progression to invasive carcinoma is significantly less common. What are the factors that cause PIN to become invasive? It appears that race and ethnicity is also an important factor! PC disproportionately affects AA men, who, along with black Jamaican men, have the highest PC incidence rates in the world. In addition, AA men develop PC significantly earlier and at the time of diagnosis they are present with the higher-grade adenocarcinoma than the age-matched EA men.
At the global level, rates of incidence are low in Asian and African men, low-to-moderate in EA men, and highest in AA men. Using data collected between 1988 and 1992, Wingo et al. reported that AAs have a 35% higher incidence rate and a 223% higher mortality rate from PC as compared with EAs. Similar data has been shown by others. The differences in incidence and mortality between AAs and EAs have been attributed to socioeconomic, environmental and biological factors, When compared with EA men, AA men present at a younger age, with higher grade (Gleason Score), and stage of disease at the onset of age, and with a greater delay in diagnosis. Whether the pathogenesis of PC is different in AA men as compared to EA men remains unanswered. Whittemore *et al*. have noted that AA men appear to have a larger volume of ‘‘latent” PC load. These investigators believe that larger-volume latent carcinomas are those that progress to become clinically evident at a faster rate, suggesting that events that account for racial differences in PC incidence may occur very early in cell transformation and thus may be genetically controlled.

Role of zinc in the pathogenesis of PC: The normal human prostate gland has an unusual capability of accumulating high levels of zinc; generally about 10-fold higher than other soft tissues. This capability resides within the mitochondrial organelles of glandular secretory epithelial cells of the peripheral zone (PZ). PZ is the main region where PC first appears. Conversely, the central and transitional zones contain relatively very low levels of zinc, except in benign prostatic hyperplasia (BPH, reviewed in. Over five decades of clinical studies have consistently demonstrated that prostate cancer tissue samples consistently contain about 65% less Zn than normal prostate tissue. More precisely, the Zn concentration (moll/g wet weight) of a normal peripheral zone tissue approximates 3000–4500; malignant peripheral zone tissue approximates one-tenth of that level (400–800); and other soft tissues approximate 200– 400. Consequently, malignant prostate tissue Zn levels are decreased by _70–85% compared to normal peripheral zone, and the decrease is observed in the glandular epithelial cells. Most importantly, one rarely, if ever, finds malignant glands that have retained the high Zn levels that characterize the normal gland. In addition, the decrease in Zn occurs early in the development of prostate malignancy. These established clinical relationships have raised important issues that relate to the role and mechanisms of Zn accumulation in the normal functioning of the prostate gland and the loss of Zn accumulation as a requirement in the development of prostate malignancy. It has been shown by Costello and Franklin groups that the functional role of Zn accumulation is to inhibit citrate oxidation of the highly specialized secretory epithelial cells, which permits the production and secretion of unusually high levels of citrate as a major component of prostatic fluid. In addition, high Zn levels in the mitochondria inhibits terminal oxidation, truncating the Krebs cycle, hence decreasing the ATP-based energy production and resulting in growth/proliferation and inducing mitochondrial apoptogenesis. And this process subsequently inhibits tumor invasion. The combination of such effects can be characterized as anti-tumor effects, which lead us to propose that Zn is a tumor-suppressor agent against prostate cancer. This provides the explanation for the requirement that malignant cells lose the capability to accumulate Zn and the basis for the absence of malignant glands that

retain high levels of zinc. This has led us to pursue the critical issues regarding the mechanism of Zn accumulation in the normal epithelial cells along with the mechanism for the lost ability of the malignant cells to accumulate zinc. The members of the Zip family of Zn transporters have been identified as important Zn transporters for the cellular uptake and accumulation of Zn in mammalian cells. More specifically, we have identified three hZIPs (hZIP1, 2, and 3) that are down-regulated. However, hZIP1 has shown to be the most important Zn uptake transporter in prostate cells.

In our present report, by utilizing well-established method of RT-ISPCR that can differentiate the relative degree of hZIP1, in different areas of the same biopsy that hZIP1 expression is significantly differentially expressed in the stromal *vs.* glandular areas and EA *vs*. AA tissues. In our recent report we demonstrated that Zn is depleted from the neoplastic as well as pre-neoplastic prostatic glandular epithelial cells. Correspondingly, hZIP1 is expressed in human normal and hyperplastic prostate glandular epithelium; but it is down-regulated in adenocarcinomatous glands. Previously, our group identified the down-regulation of hZIP1 expression in the high prostate cancer at-risk African American male population as compared with European American males in a small number of patients we tested. In this report we show down-regulation of hZIP1 in a much larger group of patients in the adenocarcinomatous glands and a significant lower expression of *hZIP1* in AA glandular epithelia as compared to the paired EA's glandular epithelia.

Relative degrees of expression of hZIP2 and hHZIP3 in the normal, BPH and malignant prostate tissues

Normal and BPH glandular epithelium consistently exhibited the strong presence of both *hZIP2* and *hHZIP3*; whereas both transporters consistently were low in the malignant glands. This represents the first report of the expression of hHZIP3 in human prostate tissue; and more importantly, reveals that *hZIP2* and *hHZIP3* are down regulated in malignant cells *in situ*. Our present studies coupled with our earlier study reveals that hZIP1, hZIP, and hZIP3 are expressed *in situ* in normal prostate glandular epithelial cells and also in hyperplastic glandular epithelium or BPH; both of which are zinc-accumulating glands. Our study confirms our previous report as well as a recent report by Costello *et al.* that identifies the expression of hZIP3 in human prostate glands. In contrast, the expression of all three transporters is consistently down-regulated in adenocarcinomatous glands, which are known to have lost the ability to accumulate zinc. These results coincide with the numerous reports that have established the consistently marked decrease in the zinc levels of prostate cancerous tissue as compared to normal and BPH prostate tissue. Thus, the expression of these *hZIP1-hZIP3* zinc uptake transporters in normal and BPH glands and the loss of their expression in malignant glands are further evidence of their role in the cellular accumulation of zinc, or lack thereof, in prostate cells. Our previous study conducted a decade ago, also showed downregulations of both *hZIP1* and *hZIP2* but in a small number of specimens (Rishi I. *et al.*, cited below).

hZIP1 provides the source of the cellular zinc. Correspondingly, the down regulation of *hZIP1* in malignant cells is consistent with their inability to accumulate zinc. If one presumes that they retain zinc uptake transporter activity in situ, their functional role might be to conserve the cellular zinc level by transporting (reabsorbing) zinc from the prostatic fluid back into the epithelial cell. Normal prostatic fluid has a high zinc concentration of ~9 mM compared to interstitial fluid zinc level of ~7 uM. At this high prostatic fluid concentration and depending upon the Km value of each transporter for zinc, *hZIP2* and *hZIP3* might effectively contribute to maintaining a high intracellular level of zinc. This implies that the down-regulation of *hZIP2* and *hZIP3* in the malignant cells would contribute to their loss of cellular zinc by the elimination of the "reabsorption" of zinc from prostatic fluid. However, it is *hZIP1* that is the major factor since its down regulation eliminates the principal source of cellular zinc; namely zinc uptake from circulation. Presently we can only speculate regarding the role of *hZIP2* and *hZIP3* in zinc accumulation by observing the degree of expressions of these two zinc transporters in normal prostate glands in situ; and the possible implications of their down regulation in the malignant glands. One of the more important observations we made is that neither of the two transporters- *hZIP2* and *hZIP3*- were significantly downregulated in AA as compared to EA.

Costello and our group both have proposed that the various effects of zinc in prostate cells constitute tumor-suppressor actions. The functional role of zinc is its prevention of citrate oxidation that is achieved by its inhibitory actions on m-aconitase activity of the highly specialized citrate-producing prostate cells. This capability now appears to result from the expression of the zinc uptake transporters that maximize the accumulation of cellular zinc. The inhibition of citrate oxidation and truncation of the Krebs cycle imposes bioenergetic and metabolic conditions under which most mammalian cells cannot survive. The high zinc levels also impose other consequential adverse effects. Zinc induces mitochondrial apoptogenesis and other effects that inhibit growth and proliferation. Obviously, the normal glandular epithelial cells under in situ conditions have evolved with mechanisms and adaptations that prevent these adverse effects. Apparently the neoplastic malignant cell is susceptible to these adverse effects of high cellular zinc. In addition, zinc also imposes inhibitory effects on mobility and invasiveness of malignant prostate cells. To avoid these effects, the neoplastic cell decreases its cellular zinc level by down regulation of the zinc uptake transporters, which is why malignant prostate glands virtually always exhibit low zinc levels in contrast to the high zinc levels that characterize normal prostate glands. In the absence of high zinc levels, the synthetic, bioenergetic, growth and proliferation, and invasive requirements of malignancy can be manifested. Consequently we propose that zinc is a tumor-suppressor agent; and *hZIP1*, along with *hZIP2* and *hZIP3*, is a tumor suppressor gene in prostate cancer.

Recently, in collaborations with Costello's group at University of Maryland, we have discovered that hZIP3 and hZIP14 zinc transporters play a pivotal role in pancreatic and hepatocellular carcinoma, respectively. These studies are ongoing in our individual laboratories.

Significance of the Findings

In the present studies, we present for the first time in a large number of the prostate core biopsy TMAs from EA Vs AA the significant ($p<0.001$) low degree of expressions of hZIP1 mRNA in the glandular epithelia of AA as compared to EA in situ by highly sensitive RT-ISPCR method.

"Task 2: To determine the differential levels of the amount and location of intracellular zinc in various areas of the prostate gland as well as in cells in different histological grades of cancer and in healthy prostate tissues by utilizing fluorescent zinc indicators and other methods."

Determination of intracellular zinc content

The relative intracellular Zn content in situ was determined only by utilizing fresh frozen tissues. Therefore, the premade TMA that we used for TASK1 could not be used for the Task 2 since the method that we described below requires active metabolic activity and for this purpose the cells must be biochemically active. The relative concentrations of Zn in various cell types of the prostatic tissues were determined according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, Oregon, USA). Briefly, the frozen tissues were incubated with equal molar concentrations of two zinc-indicator dyes: NPG and TSQ. The frozen tissues were incubated in 20 um/section of the Zn indicator cocktail over night and washed in PBS, gently, without disturbing the tissues. The slides were heat-fixed for 10 s at 104^0C to immobilize the signals. These slides were mounted with solution containing 50% glycerol in PBS and observed under a fluorescent microscope. TSQ has a selective high affinity for Zn (Kd 10 nM) and a detection limit of 0.1 nM. The ZN-TSQ positive cells stain red. NPG has moderate zinc-binding affinity (Kd _1 lM: 19). The ZN-NPG positive cells appear yellowish green. Together, TSQ and NPG provide a relative difference in Zn concentrations in various cell types of the prostate. TSQ has about 2–3-log higher affinity for Zn than NPG, but has detection limit of about 3-log lower than NPG. Therefore, the cells that contain very low concentrations of intracellular Zn appear red and the ones with higher concentrations appear green. The cells that fall in between appear yellowish or yellow-green.

Results for Task 2

1. Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral zones of the glands. In this study, Fig. 7A shows the high level of cellular Zn that characterizes the normal glandular epithelial cells (green color in Fig. 1A). In contrast, the stroma exhibits relatively lower levels of zinc. Therefore, the in situ Zn staining utilizing two different color indicators with different affinity and intracellular threshold provides the differential Zn accumulation between normal glandular epithelium and stroma. The marked reduction of cellular Zn in the epithelium of the two high grade intraepithelial neoplasia are apparent in Fig. 1B and C. Similar pattern is also seen in patient with adenocarcinoma Gleason score 3 + 3 (well differentiated) in Fig. 1D. Like the expression of hZIP1, the loss of Zn occurs early in malignancy. Due to the depletion of Zn in the malignant glands, the stromal Zn level gives the appearance of relatively higher Zn levels. Many studies have observed that Zn levels are greatly decreased in extracts of resected malignant tissue preparations , including our own [Sarafanov AJ, Todorov TI, Centeno JA, Macias V, Gao W, Liang W, Beam C, Gray MA, Kajdacsy-Balla A: Prostate Cancer Outcome and Tissue Levels of Metal Ions. *Prostate* 71:1231-8, 2011]. However, our present study provides the first in situ detection of the depleted cellular Zn levels in adenocarcinomatous glands as compared to the high Zn levels in normal glandular epithelium. Of note, the decrease in Zn level in the malignant glands is due to a decrease in the cellular accumulation of zinc. This suggests that the decrease in intracellular zinc, and not impaired secretion of Zn into the lumen (prostatic fluid), is principally responsible for the decrease in malignant tissue Zn level.

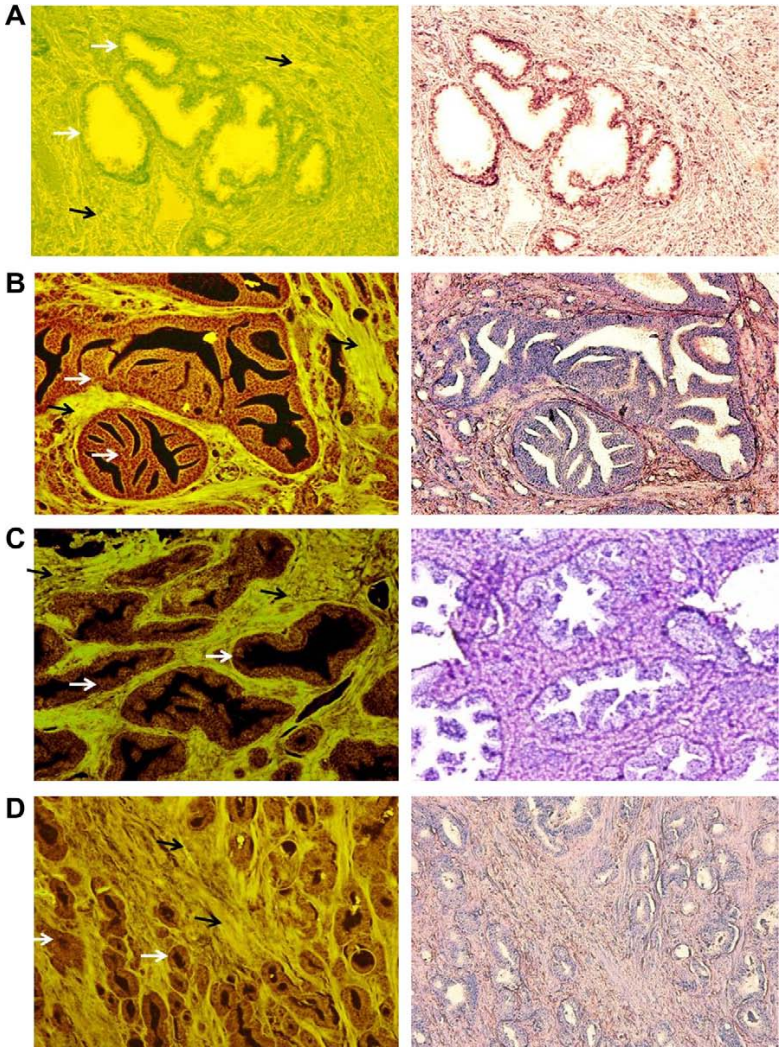


Figure 7: Zinc levels in prostate tissue frozen sections. Representative zinc levels in prostate sections. Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral zones of the glands. High Zn is represented by Newport Green, yellow/green stain and low Zn is represented by TSQ red stain. (A) Normal prostate gland from a 42 year old subject. Of note, the high level of cellular Zn indicated by dark green staining with New Port Green (NPG) Zn indicator dye that characterizes the normal glandular epithelial cells (black arrows, in A). In contrast, the stroma exhibits relatively lower levels of zinc, indicated by less intense green color in the stroma (white arrows). Therefore, the in situ Zn staining utilizing two different color indicators with different affinity and intracellular threshold provides the differential Zn accumulation between normal glandular epithelium and stroma. The marked reductions of cellular Zn in the epithelium of the two high grade intraepithelial neoplasia are shown in (B) and (C). The malignant region of the peripheral zone shows a significant depletion of Zn in the malignant glandular epithelium as exhibited by the red staining (white arrows) in three patient's resected tissues. Here, one notes relative depletion of Zn indicated by TSQ red Zn indicator dye and relatively higher levels of Zn in the stromal areas. Similar relative depletion of Zn is also observed in (D) pattern is also seen in patient with adenocarcinoma Gleason score 3 + 3 (moderately differentiated) in (D). H&E sections from parallel sections are shown on right side of the slide (final magnification 100x).

Task 3: ~~To develop antibodies against the four zinc transporters, we will carry out an extensive quality control protocol for these antibodies. We will perform immunohistochemical analyses of TMAs and determine the degree of each of the four *hZIP* protein expressions by semi-quantitative image analyses~~ (due to concerns that we would not be able to find suitable antibodies for this task, it was removed by the DOD following recommendations of the review panel).

We were able to obtain an aliquot of anti-hZIP1 polyclonal avian antibody from the Costello laboratory in Maryland. With this antibody we tested the hypothesis that hZIP1 is more highly expressed in less aggressive prostate cancers using an outcomes type TMA with 200 recurrence cases vs. 200 nonrecurrence cases in a nested case-control design. There was no difference in outcomes according to hZIP1 expression. In a limited sample of 70 subjects, of this TMA we also had zinc measurements, but there was no correlation between hZIP1 expression in the malignant epithelium and zinc levels in the tissue adjacent to the tumor (stroma and epithelium combined for ICP-MS measurements. The small number of individuals with high cadmium precludes a definitive statement.

KEY RESEARCH ACCOMPLISHMENTS

During the tenure of this grant until today, June 4, 2013, we have completed all of the key tasks, including:

- The relative degree of *hZIP1* is differentially expressed in the prostate tissues between stroma versus glandular areas. This study has been published in a peer-reviewed article).
- The *hZIP1* is downregulated in the glandular malignant tissues of the prostate.
- The *hZIP1* is also downregulated in the stromal areas in the malignant glands.
- The degree of expression downregulation is significantly ($p<0.001$) in AA as compared to EA
- The downregulation of *hZIP1* is significantly ($p<0.001$) downregulated in the glandular epithelia of AAs as compared to EAs.
- *hZIP2 and hZIP3* are also downregulated in the adenocarcinoma of both EA and AA but there were no significant differences between the two ethnic groups with regards to degrees of expressions of these two zinc transporters.
- *hZIP4* appears to be absent in the prostate glands.
- Zinc accumulation is depleted in the glandular regions of adenocarcinoma of prostate as compared to normal tissues. With the current technology we are unable to determine the degrees of zinc depletion in EA Vs AA.

- We discovered the microRNA cluster that regulates mRNA levels of *hZIP1* and other zinc transporters. The initial data, with 5 patients for each ethnic group shows that here is a strong inverse correlation between this cluster and of microRNAs and *hZIP1* in EAs, but not in AAs (published JBC: Appendix 2). Dr. Larisa Nonn, a junior faculty at Dr. Balla's department at UIC was the senior author of this paper. She went on with this project and now has an RO1 grant from the NCI to further study the role of microRNAs in the regulation of intracellular zinc.
- hZIP1 expression by immunohistochemistry in cancer cells is not a prognostic marker in a large cohort of 400 prostatectomy subjects (nested case control TMA).
- In a relative small cohort of 70 subjects hZIP1 expression by immunohistochemistry in cancer cells does not correlate with zinc concentrations in normal tissues adjacent to cancer when ICP-MS is used by whole tissue grinding.
- In part funded by this grant, we published in *Prostate* in 2010 a study that showed worse prognosis for cancer recurrence after prostatectomy when zinc concentrations are low in normal appearing tissues adjacent to prostate cancer.

REPORTABLE OUTCOMES

a) Publications

Johnson LA, M A. Kanak, A Kajdacsy-Balla, J P. Pestaner, and O Bagasra. Differential Zinc Accumulation and Expression of human Zinc Transporter 1 (*hZIP1*) in Prostate Glands. **Methods.** 2010; 52:316-321.

Costello, L; Levy, B; Desouki, M; Zou, J; Bagasra O; Johnson, L; Hanna, N; Franklin, R. Decreased Zinc and Down Regulation of ZIP3 Zinc Uptake Transporter in the Development of Pancreatic Adenocarcinoma. **Cancer Biol Ther.** 2011; 12:297-303.

Nonn L, Khramtsova E, Mihelich B, Arva N, Vaishnav A, Johnson D, Antonio A, Martens-Uzunova E, O Bagasra, Kajdacsy-Balla A. Prostatic zinc homeostasis is regulated by the Mir-183-96-182 cluster. **J. Biol Chemistry**, 2011; 30:286:44503-11.

Sarafanov AJ, Todorov TI, Centeno JA, Macias V, Gao W, Liang W, Beam C, Gray MA, Kajdacsy-Balla A: Prostate Cancer Outcome and Tissue Levels of Metal Ions. **Prostate** 2011; 71:1231-8,

Franklin RB, Levy BA, Zou J, Hanna N, Desouki MM, Bagasra O, Johnson LA, Costello LC. ZIP14 Zinc Transporter Downregulation and Zinc Depletion in the Development and Progression of Hepatocellular Cancer. **J Gastrointest Cancer.** 2012;43:249-57.

Note: The most comprehensive manuscript about this work is still in preparation. It will have the results of hZIPs expression in EA and AE.

b) Abstracts

Johnson LA, K Berry, *O.* Bagasra. Health disparities among African Americans: the role of zinc in pathogenesis of prostate cancer. AACR: The Science of Cancer health Disparities. Abstract # B55. Feb 3-6, 2009.

Johnson Leslie (2009). Molecular Pathogenesis of Prostate Cancer in Relation to the African American Community, the Role of Zinc and Zinc Transporters: Environment and Genetic Influences. 2nd Annual James E. Clyburn Health Disparities Lecture: Social Determinants of Health: Framing the Issues at the University of South Carolina in Columbia, SC

Leslie A. Johnson, Kendall Williams, Keaira Berry, Jacob Sterling, Andrea' Kajdacsy-Balla and Omar Bagasra. Differential Expression of *ZIP1* in nonmalignant prostate tissue: Racial Differences. 3rd Annual Prostate Cancer Conference, March 14-16, 2010, Atlanta GA.

Keaira Berry, Omar Bagasra, MD, PhD, Leslie Johnson. The Role of Zinc in the Early Detection of Prostate Cancer.. Claflin University Export Program. July 11, 2008.

Keaira Berry, Omar Bagasra, MD, PhD, Leslie Johnson The Role of Zinc in the Early Detection of Prostate Cancer Annual Biomedical Orlando, Florida November 5- 8, 2008 Abstract B71. Page 137. Research Conference for Minority Students (*Won ABRCMS Presentation Award for 2008).

Keaira Berry, Bethany McGonnigal, James Padbury MD. Placental LAT-1 Expression in Healthy and Adverse Pregnancies Abstract G3. Page 364 Annual Biomedical Research Conference for Minority Students Phoenix, Arizona November 4- 7, 2009.

Keaira Berry, Leslie A. Johnson, Kendall Williams, Andre Kajdacsy-Balla, Omar Bagasra Differential expression of Human Zinc Transporters 1 (hZIP1) in nonmalignant prostate tissue: Racial Differences 3rd Annual James E. Clyburn Lecture Series University of South Carolina, Columbia April 9, 2010.

Keaira Berry, Leslie A. Johnson, Kendall Williams, Andre Kajdacsy-Balla, Omar Bagasra: Differential expression of Human Zinc Transporters 1 (hZIP1) in nonmalignant prostate tissue: Racial Differences Nobel Laureate Chemistry (Dr. Martin Chalfie) Lecture Series Claflin University, Orangeburg, SC April 20, 2010.

Bianca Thomas, Jazzmine Clemons, Kendall Williams, Leslie A. Johnson, Joseph P. Pestner, Omar Bagasra. Differential expressions of zinc transporters in β-cells in African Americans. 3rd Annual James E. Clyburn Lecture Series University of South Carolina, Columbia. April 9, 2010.

Bianca N. Thomas, Leslie A. Johnson, Jacob Sterling, and Dr. Omar Bagasra. The Effects of Zinc Accumulation pertaining to Diabetes in the African American community vs. other racial populations Claflin University, Orangeburg, SC March 27, 20102nd Annual Open House & Research Day (*won Second Prize in Poster Competition).

Jessica Abercrombie, Leslie A. Johnson, Kendall M. Williams, and Dr. Omar Bagasra. The Molecular Relationship between Zinc and the Breast Epithelium: African Americans vs other Racial Populations2nd Annual Open House & Research Day. Claflin University, Orangeburg, SC March 27, 2010.

Clara L. Jones, Jessica Abercrombie, Leslie A. Johnson, Kendall Williams, Joseph P. Pestner, & Dr. Omar Bagasra Role of Zinc Transporters and the Role of Zinc in Breast Epithelia of various racial groups. 3rd Annual James E. Clyburn Lecture Series University of South Carolina, Columbia April 9, 2010.

Clara L. Jones, Jessica Abercrombie, Leslie A. Johnson, Kendall Williams, Joseph P. Pestner, & Dr. Omar Bagasra Role of Zinc Transporters and the Role of Zinc in Breast Epithelia of various racial groups Nobel Laureate Chemistry (Dr. Martin Chalfie) Lecture Series Claflin University, Orangeburg, SC April 20, 2010.

Keaira Berry, Leslie A. Johnson, Kendall Williams, Andre' Kajdacsy-Balla, Omar Bagasra Differential expression of Human Zinc Transporters 1 (hZIP1) in nonmalignant prostate tissue: Racial Differences Nobel Laureate Chemistry (Dr. Martin Chalfie) Lecture Series Claflin University, Orangeburg, SC April 20, 2010.

Clara L. Jones, Jessica Abercrombie, Leslie A. Johnson, Kendall Williams, Joseph P. Pestner, & Dr. Omar Bagasra Role of Zinc Transporters and the Role of Zinc in Breast Epithelia: evaluating the molecular pathogenesis of health disparities in breast cancer. The 5th Annual Texas Conference on Health Disparities. Fort Worth, TX May 27-28. 2010.Abst #132.

Leslie A. Johnson, Keaira Berry Kendall Williams, Andre' Kajdacsy-Balla, Omar Bagasra. Prostate Cancer: A health disparity among African American men-The racial differences within nonmalignant prostate tissue pertaining to the differential expression of *hZIP1*. Third AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Miami, FL Sept 30-Oct 3rd. 2010 (Abst # 285).

Clara L. Jones, Leslie A. Johnson, Joseph P. Pestaner, & Dr. Omar Bagasra. Zinc transporter activation and the later age of lactation may increase the risk for breast cancer. . Third AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Miami, FL Sept 30-Oct 3rd. 2010 (Abst # 140).

Keaira Berry- G110- Prostate cancer: A health disparity among African American men differential expression of hZIP1 within nonmalignant prostate tissue reveals the role of genes and environment. ABRCAMS Charlotte, 2010 Nov 9-13 ABSTRACT #-G110.

Jones C L. Molecular pathogenesis of breast cancer: Differential expression of zinc transporters in breast tissues from various ethnic groups. ABRCAMS Charlotte, 2010 Nov 9-13 ABSTRACT #-G43.

Thomas B, O Bagasra. The role of zinc and zinc transporters in the pathogenesis of Diabetes Mellitus: Health disparity addressed on the bases of race and the environment. ABRCAMS Charlotte, 2010 Nov 9-13, ABSTRACT #-A40.

Howard K, Johnson LA, Bagasra O. Differential Expressions of EGFR, VEFGA, PLAU, and MAP2K7 in African American and European American Primary Breast Cancer Cell Lines by PCR Array. AACR: Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications Special Conference October 12-15, 2011, San Francisco, CA, Abstract# 260207.

Howard K, Bagasra O. Differential Expressions of Four Genes Linked to Breast Cancer and the Regulatory Role of Homologous miRNAs. ABRCMS. November 9-12, 2011, St. Louis, MO. Abstract # D010. Khirston Howard, Leslie Johnson, Omar Bagasra MD. Ph.D. Differential Gene Expressions in Breast Cancer Epithelia of African American and European American Women: Health Disparity at the Molecular Level (2012 NIH Summit on Health Disparity, Nov 1, 2012

Kajdacsy-Balla A, Nonn L, Arva N, Macias V, Shah J, Nejati R, Sarafanov A,. Centeno JA, Todorov T, Bagasra O: Prostate low zinc concentration: Prognostic implications for prostate cancer, relation to hZIP1 zinc transporter expression and its regulator micro RNAs. IMPaCT 2011- Innovative Minds in Prostate Cancer Today, Orlando, FL, March 9-12, 2011

Khramtsova EA, Arva NC, Vaishnav A, Bagasra O, Kajdacsy- Balla A, Nonn L: Disease- and race-related differences in microRNAs and zinc transporter 1 in human prostate tissue. AACR 101st Annual Meeting, Washington, DC, April17-21, 2010

Sarafanov AJ, Todorov TI, Centeno JA, Macias V, Gray MA, Gao W, Liang W-M, Beam C, Kajdacsy-Balla A: Prostate cancer outcome and tissue levels of metal ions. AACR 100th Annual Meeting, Denver, April 22, 2009

c) Presentations

Kajdacsy-Balla A: Prostate cancer outcome and tissue levels of metal ions". Invited lecture at the XXIX International Congress of the Academy of Pathology and World Congress of Academic and Environmental Pathology. October 4, 2012. Cape Town, South Africa.

d) Funding applied for based on work supported by this award

NIH: "MiR-183-96-182 cluster, prostatic zinc homeostasis and carcinogenesis"

PI: Larisa Noon Co-I: Andre Kajdacsy-Balla and O. Bagasra

Period: 2012-2017 $2.2M/5 yrs

CONCLUSION

This was a Synergy grant to Omar Bagasra's laboratory (Claflin University, Orangeburg, SC) and my laboratory (University of Illinois at Chicago, IL). We have been working on the differential expressions of zinc transporters, especially human zinc transporters 1-4 (hZIP1-4) for the last five years. Most particularly we have been looking at the zinc transporters that may be disparately expressed in the prostate tissues of African American (AA) males as compared to European American (EA) males using the CPCTR Ethnicity TMAs that we got from you. This may lead to the identification of potential molecular targets for nutritional, environmental or life style factors for their potential relationship to the incidence or progression of prostate cancer in various racial groups. The TMAs were analyzed for differential expressions of *hZIP1-hZIP4* mRNA expressions *in situ by RT-in situ PCR*. A significant ($p<0.001$) differential down-regulations of *hZIP1* were recorded for AAs as compared to EAs. We did not record any significant differences in the relative expressions of other three hZIPs (hZIP2-4).

These findings with hZIP1 are important. They tell us that AA are at a disadvantage when it comes to zinc transport into cells. We and others have shown that low zinc in the prostatic tissue is associated with worse prognosis. hZIP1 is the major regulator of zinc intake, and lower *hZIP1*expression may be one of the explanations for worse prognosis in AA. Confirmation by hZIP1 protein expression is still necessary for this conclusion, and this is the subject of our current efforts.

One cannot jump to the conclusion that zinc supplementation is useful or even safe for patients with prostate cancer. In our future work, we would like to address this issue. The work funded by this DOD grant indicates that AA may have a reason for low tissue zinc levels.

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Costello, L; Levy, B; Desouki, M; Zou, J; Bagasra O; Johnson, L; Hanna, N; Franklin, R. Decreased Zinc and Down Regulation of ZIP3 Zinc Uptake Transporter in the Development of Pancreatic Adenocarcinoma. **Cancer Biol Ther.** 2011;12:297-303.

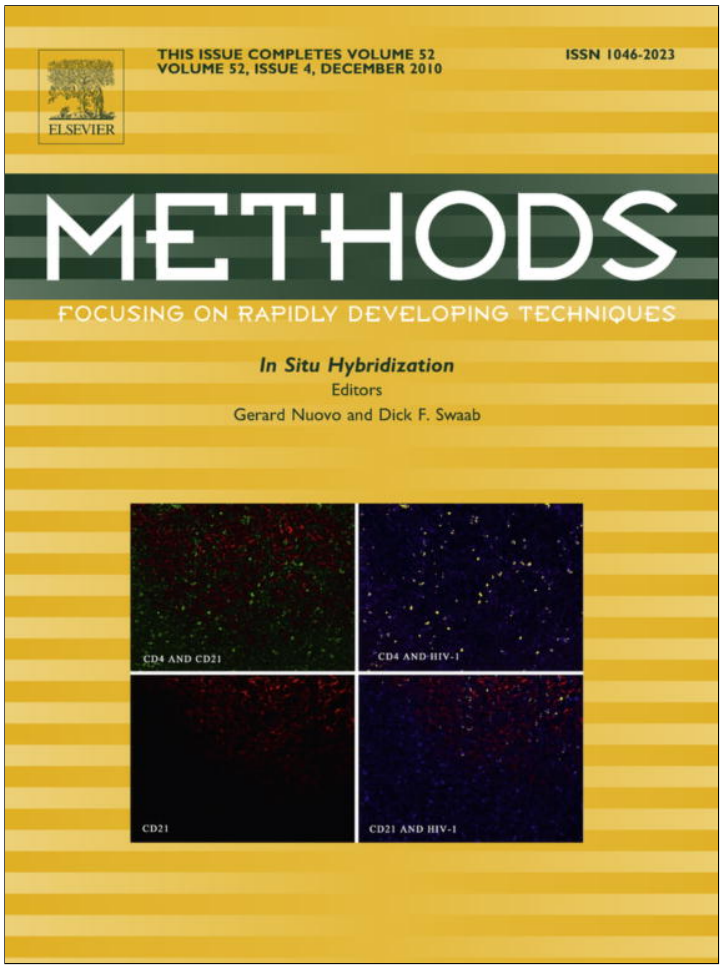
Nonn L, Khramtsova E, Mihelich B, Arva N, Vaishnav A, Johnson D, Antonio A, Martens-Uzunova E, O Bagasra, Kajdacsy-Balla A. Prostatic zinc homeostasis is regulated by the Mir-183-96-182 cluster. **J. Biol Chemistry**, 30;286:44503-11.

Franklin RB, Levy BA, Zou J, Hanna N, Desouki MM, Bagasra O, Johnson LA, Costello LC. ZIP14 Zinc Transporter Downregulation and Zinc Depletion in the Development and Progression of Hepatocellular Cancer. **J Gastrointest Cancer.** 2012 Jun;43:249-57.

Sarafanov AJ, Todorov TI, Centeno JA, Macias V, Gao W, Liang W, Beam C, Gray MA, Kajdacsy-Balla A: Prostate Cancer Outcome and Tissue Levels of Metal Ions. **Prostate** 71:1231-8, 2011

APPENDICES

The three publications which are most relevant to this grant are presented below (Johnson LA *et al*, 2010; Nonn L *et al*., 2011; Sarafanov AJ *et al*., 2010).



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Differential zinc accumulation and expression of human zinc transporter 1 (*hZIP1*) in prostate glands

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ABSTRACT

Zinc (Zn) is essential for a very large number and variety of cellular functions but is also potentially toxic. Zn homeostasis is therefore dynamically maintained by a variety of transporters and other proteins distributed in distinct cellular and subcellular compartments. Zn transport is mediated by two major protein families: the Zip family, which mediates Zn influx, and the ZnTs which are primarily linked to Zn sequestration into intracellular compartments and are, thereby, involved in lowering cytoplasmic Zn free ion concentrations. In the prostate epithelial cell, the accumulation of high cellular zinc is a specialized function that is necessary for these cells to carry out the major physiological functions of production and secretion of prostatic fluids. The loss of Zn accumulation is the most consistent and persistent characteristic of prostate malignancy. Currently, there are no direct methods to determine the relative Zn levels in various cell types of prostate gland (i.e. stroma, glandular epithelia, acini, and muscular) and no reliable ways to compare the Zn in normal versus malignant areas of the gland. Here we report a new method to show a differential Zn staining method that correlates with various stages of prostate cancer development *in situ* and expression of a human Zn transporter1-*hZIP1* – *in situ* by *in situ* reverse transcriptase-polymerase chain reaction hybridization (ISRTPCR) that correlate with the relative Zn levels determined by the differential Zn staining method. By utilizing these methods, we show for the first time that: (1) the relative Zn levels are very low to absent in the malignant glands, (2) normal glands show high Zn levels in both glandular epithelia as well as in stromal tissues, (3) the Zn levels begin to decrease in pre-malignant glands and precedes the development of malignancy, and (4) the expression of human Zn transporter1 (*hZIP1*) appears to correlate with the Zn levels in the prostate glands and may be the major Zn regulator in this organ.

1. Introduction

The role of Zn, its underlying active function in the development and progression of prostate malignancy and its potential application in the prevention and treatment of prostate cancer are critical issues for the medical/scientific community and the public-at-large [1]. Prostate cancer (PC) is the second most common form of cancer diagnosed in men [1,2] and it is the most prevalent type of cancer observed in African American (AA) men [3–5]. Very early detection is the key to effective treatment of PC and to the prevention of deaths due to the progression of untreatable advanced stages of cancer. Mitigating factors, especially benign prostatic hyperplasia (BPH), result in a low accuracy (about 60%) of prostate-specific antigen (PSA) testing. Thus, there is an urgent need for a more reliable biomarker to identify PC at a very early stage and to identify 'at-risk' individuals. AAs are at significantly higher risk of developing PC particularly at an earlier age and have a more aggressive form of PC than European American (EA) men [1,2]. Currently, there are no satisfactory ways to differentiate between Stages I/II indolent and lethal (aggressive) PC at diagnosis. Both early identification and indolent/lethal differentiation are critical because PC, if identified while confined to the prostate, (a) is "curable" for aggressive tumors by surgery and subsequent treatment and (b) "watchful waiting" would be appropriate for indolent tumors [1–3].

Numerous studies consistently have shown that the Zn levels of malignant prostate tissue are significantly decreased as compared to the normal prostate tissue (reviewed in [6,7]). This consistency persists in different reports by different investigators employing different populations and tissue samples and involving various stages of malignancy. The studies of Zaichick et al. [8], Vartsky

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et al. [9], and Franklin et al. [7] further reveal the critically important relationship that, in individual analyses, malignant prostate tissue always exhibits relatively low Zn levels as compared to the normal tissues. In addition, Habib [11] reported that the decrease in Zn occurs early in malignancy. These persistent results, and the additional corroborating evidence presented previously (reviewed in [6,7]), has firmly established that the unique zinc-accumulating capability of the normal peripheral zone secretory epithelial cells is lost in the neoplastic transformation to malignant cells [6,7,12–14].

This study was done in order to determine if Zn molecules are differentially accumulated in various cell types and *hZIP1* is the major Zn transporter that regulates Zn in prostate glands, as previously proposed [7,15]. In order to determine if Zn accumulation in the glandular portions of the prostate is significantly different than the Zn in the stromal and other cell types, we evaluated prostatic resections from 19 men with prostate cancer and four normal prostatic tissues and evaluated their tissues for the differential Zn accumulation by utilizing two Zn indicator dyes: New Port Green DCF (NPG) and *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ) to determine the relative Zn concentrations in various histological cell types, *in situ*. In addition, we also explore the differential expression of *hZIP1* by *in situ* RT-PCR method (ISRTPCR) using the same group of patients.

2. Methods and materials

2.1. Human subjects and study protocol

Fresh frozen specimens of primary prostate carcinoma were received from the University of Illinois, Department of Pathology (Dr. Balla) on dry ice, according to the approved protocol of the institutional review board as Claflin University IRB. Normal prostate tissues were autopsy specimens obtained from the Department of Pathology, Brody School of Medicine, East Carolina University, Greenville, NC (Dr. Pestaner).

2.2. In situ RT-PCR of human tissue sections

Fresh frozen sections from 19 male prostate biopsies with a clinical history of prostate cancer, and 6 from autopsy specimens with normal glands who died from automobile accidents, were processed for RT-*in situ*-PCR [16,17]. All reagents for the continuation of the experiment were prepared in RNase-free H_2O. Briefly, fresh frozen tissue sections were received from each of our collaborators in a blinded fashion. All slides were fixed in 2% paraformaldehyde solution, overnight for a 24 h period. After fixation, slides were washed in 3× PBS once, and then twice in 1× PBS. These slides were then treated with proteinase K (6 μg/ml) at room temperature for 23 min. Proteinase K was inactivated by incubating slides on a heat block at 95 °C for 2 min. To perform the amplification of mRNA sequences for *hZIP1*, we used multiple spliced sequences that flank the junctions of two exon splice sites. Because these RNA-specific primers will not amplify the genomic DNA template, one can perform the amplification of multiple mRNAs simultaneously. The following primer pairs were used for amplification: sense 5′-TCAGAGCCTCCAGTGCCTGT-3′ and antisense 5′-TTGTCT CCTGGACCTGCTGC-3′ for *hZIP1* that gave a 189-bp product. To form cDNA copy of the template and to amplify, we used one-step *in situ* RT-PCR enzyme – *rTth* enzyme, which has both the RT and polymerase function. The amplification cocktail contained the pair of primers at 100 pM each in 50 mM Tris, pH 8.3, 8.5 mM MgCl, 10 mM $MnCl_2$, 40 mM KCL, 1 mM dithiothreitol, 10× transcription buffer, 10× chelating buffer, 5 U *rTth* (recombinant thermostable DNA polymerase) enzyme. After amplification, slides were washed in 2× SSC buffer, three times and then amplicons were detected by *in situ* hybridization method [16,17].

Hybridizations were performed with 41 bp FITC (fluorescein isothiocynate) oligonucleotide probe for *hZIP1* (oligonucleotide: 5′-FITC-CCTGGTGCCCATCTGTGTGCTGCGC-CGGCCAGGAGCTAAC-3′). Hybridizations were performed in a buffer containing 50% formaldehyde, 10 mM dithiothreitol, 2× sodium chloride/sodium citrate solution, 100 μg/mL fragmented salmon sperm DNA, 2% bovine serum albumin, 1 mg/mL *Escherichia coli* tRNA, and 20 pmol probes at 95 °C for 2 min, then 45 °C for 18 h. These tissue sections were then washed to remove unbound probes and viewed under UV-epifluorescence microscope after the cells were washed. To preserve the intensity of the hybridized probes, the tissues were not counter-stained. Parallel hematoxylin and eosin-stained slides were used to identify various histologic cell types in the tissue sections. Since we utilized frozen section the H&E parallel sections are not as crisp and sometimes difficult to identify due to tissue damage during cutting. Microscopic examination usually reveals cytoplasmic staining for mRNA versus nuclear staining for DNA. Cell enumeration was performed on coded slides by at least two pathologists. Twenty microliters of RT (reverse transcriptase enzyme) cocktail was added to each slide. The slides were then sealed with slide frame sealer and inserted into the slide slots of a thermocycler specially designed for *in situ* PCR (MJR/Bio-Rod – Twin Tower, PTC 200, Waltham, MA). The two cycles were programmed for 30 min at 50 °C, then 95 °C for 5 min (for cDNA step), and then cDNAs were amplified for 30 cycles at 95 °C denaturing, 61.7 °C annealing, and 72 °C extension.

2.3. Determination of intracellular zinc content

The relative intracellular Zn content *in situ* was determined by utilizing fresh frozen tissues. For this purpose the cells must be biochemically active. The relative concentrations of Zn in various cell types of the prostatic tissues were determined according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, Oregon, USA). Briefly, the frozen tissues were incubated with equal molar concentrations of two zinc-indicator dyes: NPG and TSQ [18,19]. The frozen tissues were incubated in 20 μl/section of the Zn indicator cocktail over night and washed in PBS, gently, without disturbing the tissues. The slides were heat-fixed for 10 s at 104 °C to immobilize the signals. These slides were mounted with solution containing 50% glycerol in PBS and observed under a fluorescent microscope. TSQ has a selective high affinity for Zn (K_d ~10 nM) and a detection limit of ~0.1 nM. The ZN-TSQ positive cells stain red [18]. NPG has moderate zinc-binding affinity (K_d ~1 μM: 19). The ZN-NPG positive cells appear yellowish green. Together, TSQ and NPG provide a relative difference in Zn concentrations in various cell types of the prostate. TSQ has about 2–3-log higher affinity for Zn than NPG, but has detection limit of about 3-log lower than NPG. Therefore, the cells that contain very low concentrations of intracellular Zn appear red and the ones with higher concentrations appear green. The cells that fall in between appear yellowish or yellow-green.

3. Results

Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral zones of the glands. In this study, Fig. 1A shows the high level of cellular Zn that characterizes the normal glandular epithelial cells (green color in Fig. 1A). In contrast, the stroma exhibits relatively lower levels of zinc. Therefore, the *in situ* Zn staining utilizing two different color indicators with different affinity and intracellular

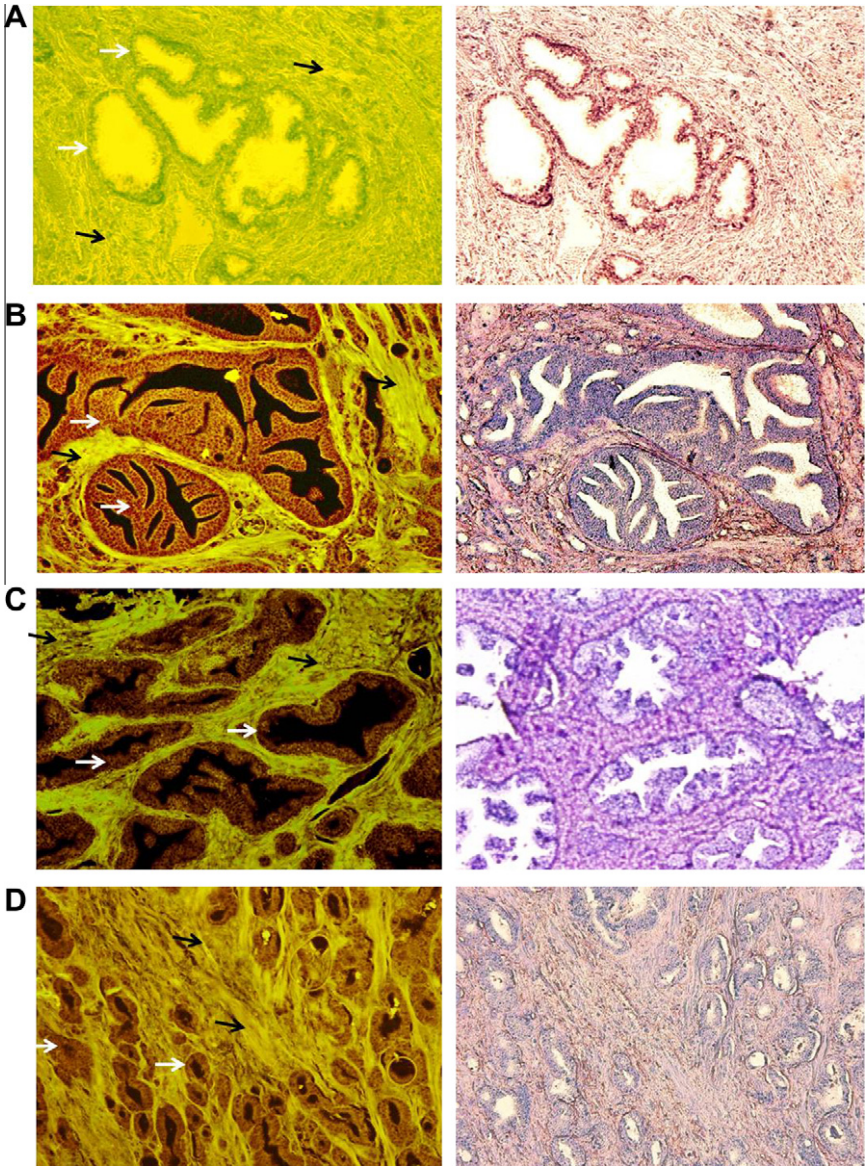

Fig. 1. Zinc levels in prostate tissue frozen sections. Representative zinc levels in prostate sections. Fresh frozen tissues were utilized to determine the relative intracellular Zn levels in various histological areas of the prostate glands. All prostate sections were from the peripheral zones of the glands. High Zn is represented by Newport Green, yellow/green stain and low Zn is represented by TSQ red stain. (A) Normal prostate gland from a 42 year old subject. Of note, the high level of cellular Zn indicated by dark green staining with New Port Green (NPG) Zn indicator dye that characterizes the normal glandular epithelial cells (black arrows, in A). In contrast, the stroma exhibits relatively lower levels of zinc, indicated by less intense green color in the stroma (white arrows). Therefore, the *in situ* Zn staining utilizing two different color indicators with different affinity and intracellular threshold provides the differential Zn accumulation between normal glandular epithelium and stroma [18,19]. The marked reductions of cellular Zn in the epithelium of the two high grade intraepithelial neoplasia are shown in (B) and (C). The malignant region of the peripheral zone shows a significant depletion of Zn in the malignant glandular epithelium as exhibited by the red staining (white arrows) in three patient's resected tissues. Here, one notes relative depletion of Zn indicated by TSQ red Zn indicator dye and relatively higher levels of Zn in the stromal areas. Similar relative depletion of Zn is also observed in (D) pattern is also seen in patient with adenocarcinoma Gleason score 3 + 3 (moderately differentiated) in (D). H&E sections from parallel sections are shown on right side of the slide (final magnification 100×).

threshold provides the differential Zn accumulation between normal glandular epithelium and stroma [18,19]. The marked reduction of cellular Zn in the epithelium of the two high grade intraepithelial neoplasia are apparent in Fig. 1B and C. Similar pattern is also seen in patient with adenocarcinoma Gleason score 3 + 3 (moderately differentiated) in Fig. 1D. Like the expression of *hZIP1*, the loss of Zn occurs early in malignancy. Due to the depletion of Zn in the malignant glands, the stromal Zn level gives the appearance of relatively higher Zn levels. Many studies have observed that Zn levels are greatly decreased in extracts of resected malignant tissue preparations [20]. However, our present study provides the first *in situ* detection of the depleted cellular Zn levels in adenocarcinomatous glands as compared to the high Zn levels in normal glandular epithelium. Of note, the decrease in Zn level in the malignant glands is due to a decrease in the cellular accumulation of zinc. This suggests that the decrease in intracellular zinc, and not impaired secretion of Zn into the lumen (prostatic fluid), is principally responsible for the decrease in malignant tissue Zn level. Thus, the results of our study are consistent with previous studies [6–10,21].

Correspondingly, Fig. 2 the relative expression of mRNA expression for *hZIP1* were determined in the 19 prostate resections. The typical results represented in Fig. 2 were consistently observed in the frozen sections of all 19 prostate resections. The results show that *hZIP1* gene expression is evident uniformly in the epithelium of the normal peripheral zone glands and is relatively low in the stroma (Fig. 2A). *hZIP1* expression is markedly down-regulated to the extent of not being demonstrable in the two high grade

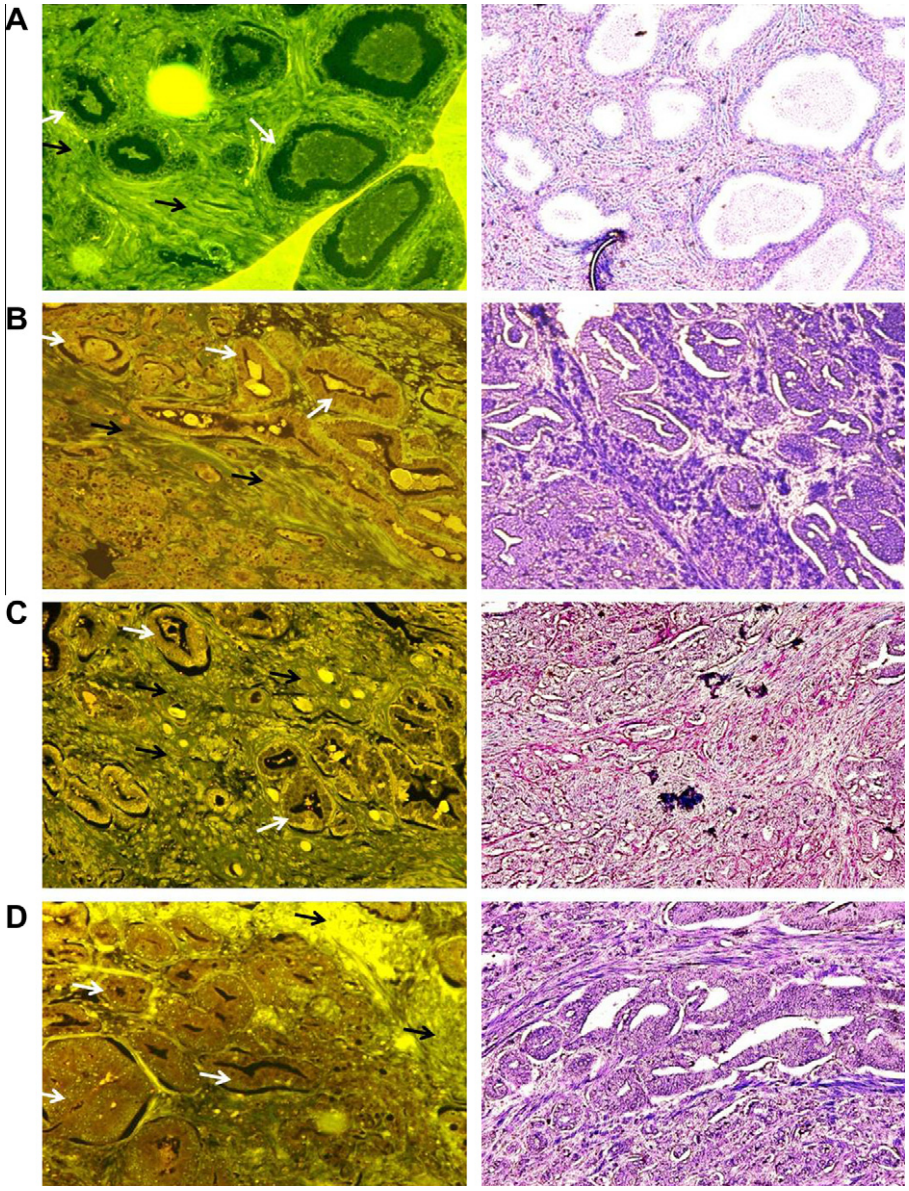

Fig. 2. (A–C) *In situ* detection of *hZIP1* mRNA expression levels in frozen prostate sections from one normal (Fig. 1A) and three prostate cancer subjects (B–D) are shown. (A) Relatively high expression of *hZIP1* in a normal prostate section, both in the glandular as well as in the stromal areas. This figure shows the relative degree of expression of *hZIP1* as determined by *in situ* RT-PCR/hybridization method. As one notes that in all three specimens from malignant tissues (B–D, white arrows), the malignant areas of the prostate, represented by abnormal glandular epithelia, exhibits a significant down-regulation of *hZIP1* mRNA as compared to the surrounding stromal areas showing a relatively higher degree of *hZIP1* expression (black arrows). H&E sections from the same specimens are shown on right side of the slide (final magnification 100×).

adenocarcinomatous glands (red colors of the glandular epithelia in Fig. 2B–C) and in moderately differentiated adenocarcinoma glands (Fig. 2D); however, it is present in the stromal tissues but at much lower levels as compared to the normal control (Fig. 2A).

Fig. 3 shows the *hZIP1* expression profiles in the frozen sections of the normal glands adjacent to malignant glands in two of the patients (Fig. 3A and B). As one can observe in Fig. 3A and B, on the right side of each slide there are mostly normal appearing glands that exhibit relatively strong yellow/green staining for *hZIP1* expression and as one moves toward the left the degree of expression of *hZIP1* decreases as the tumor grade of adenocarcinomatous glands begin to increase. In the same patient (Fig. 3B) as one moves further to the left (Fig. 3C), one can easily recognize lower grade tumor and relatively higher degree of *hZIP1* expression. In this section, one can also note the overall increase in the relative *hZIP1* expression.

4. Discussion

Worldwide, there are more than 10 million new cancer cases each year, and cancer is the cause of approximately 12% of all deaths. Among all cancers, PC is the second leading cause of male cancer related deaths [22,23]. Over 200,000 males were identified with PC in 2003 and as a result ~30,000 died. In 2010 more than 186,000 US men will be diagnosed with PC and over 30,000 may die. Despite the extensive clinical and experimental studies over the recent decades, the pathogenesis of PC remains unanswered (reviewed in [1,2]). The interaction of genetics and the environment and its influence on the molecular mechanisms responsible in the development and progression of malignant prostate cells are largely unknown [24–26]. There is a great need to explore the role of differential gene expression that leads to altered cellular metabolism as an essential factor in prostate malignancy [27]. The

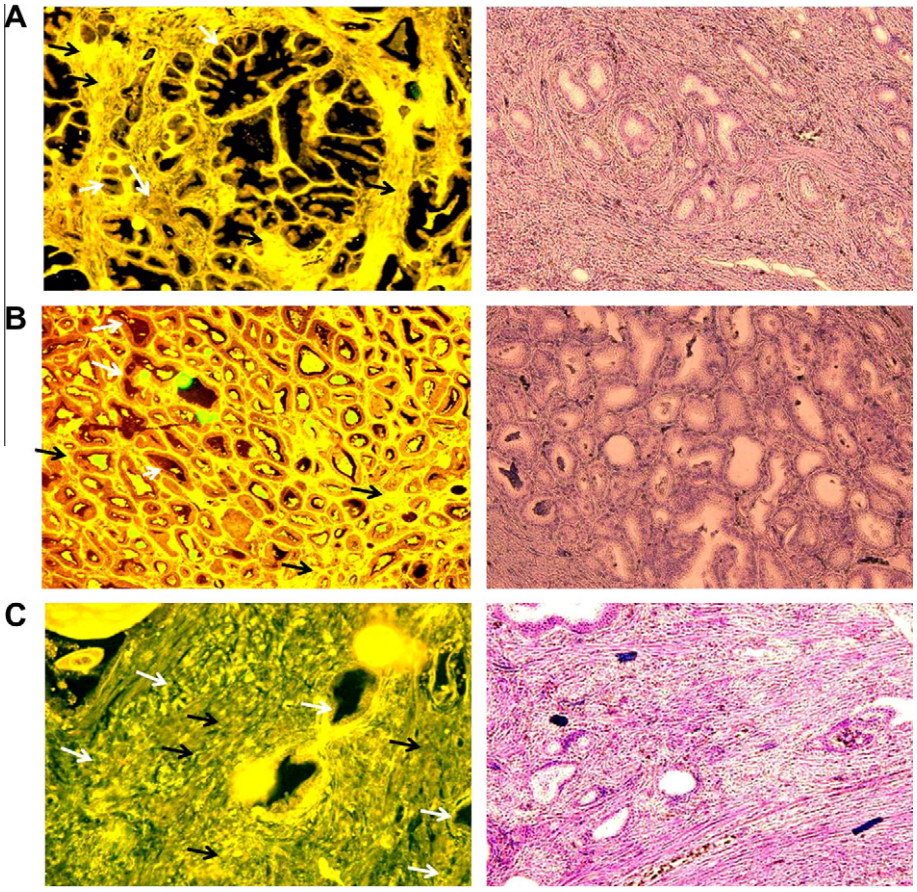

Fig. 3. *In situ* detection of *hZIP1 mRNA* and the frozen sections of normal and malignant glands in the same tissue sections. (A and B) Analyses of *hZIP1* expression by *in situ* RT-PCR/hybridization are shown from two patients. Relatively high levels of *hZIP1* expressions in normal appearing glands can be seen as a yellowish/green color on the right portions (white arrows) of the slides whereas low or absent expression can be seen as red colors in malignant glands on the left sides of the slides (black arrows). Of note, the greenish color in the stoma in (A) and (B) are absent, suggesting low expression of *hZIP1*. In the same patient (B) as one further moves to the left (C) towards relatively normal appearing area, one can easily recognize higher expression of *hZIP1* (shown in greenish color and more normal appearing glands; black arrows). H&E sections from the same specimens are shown on right side of the slide (final magnification 100×).

combination of genetic/molecular/environmental factors and their relationships are required to identify the critical events in the prostate malignancy process. Such studies are proving to be very useful in the understanding of the molecular pathogenesis of prostate cancer [6,7,28].

Zinc and Zn transporters play an important role in the molecular pathogenesis of PC [6,7,20,21]. PC afflicts one out of nine men over the age of 65 years. Prostatic intraepithelial neoplasia (PINs) is relatively common and occurs early in life [1,2]. However, progression to invasive carcinoma is significantly less common. What are the factors that cause PIN to become invasive? It appears that race and ethnicity is also an important factor! PC disproportionately affects AA men, who, along with black Jamaican men, have the highest PC incidence rates in the world [22,23,29–34]. In addition, AA men develop PC significantly earlier and at the time of diagnosis they are present with the higher-grade adenocarcinoma than the age-matched EA men [31–35].

At the global level, rates of incidence are low in Asian and African men, low-to-moderate in EA men, and highest in AA men [22,23,30,32]. Using data collected between 1988 and 1992, Wingo et al. [36] reported that AAs have a 35% higher incidence rate and a 223% higher mortality rate from PC as compared with EAs. Similar data has been shown by others [22,23]. The differences in incidence and mortality between AAs and EAs have been attributed to both environmental and biological factors [6,7,27–29], When compared with EA men, AA men present at a younger age, with higher grade (Gleason Score), and stage of disease at the onset of age, and with a greater delay in diagnosis [37–39]. Whether the pathogenesis of PC is different in AA men as compared to EA men remains unanswered. Whittemore et al. [40] have noted that AA men appear to have a larger volume of "latent" PC load. These investigators believe that larger-volume latent carcinomas are those that progress to become clinically evident at a faster rate, suggesting that events that account for racial differences in PC incidence may occur very early in cell transformation and thus may be genetically controlled [33,35].

4.1. Role of zinc in the pathogenesis of PC

The normal human prostate gland has an unusual capability of accumulating high levels of zinc; generally about 10-fold higher than other soft tissues. This capability resides within the mitochondrial organelles of glandular secretory epithelial cells of the peripheral zone (PZ). PZ is the main region where PC first appears. Conversely, the central and transitional zones contain relatively very low levels of zinc, except in benign prostatic hyperplasia (BPH, reviewed in [6,7,8,10]). Over five decades of clinical studies have consistently demonstrated that prostate cancer tissue samples consistently contain about 65% less Zn than normal prostate tissue. More precisely, the Zn concentration (nmol/g wet weight) of a normal peripheral zone tissue approximates 3000–4500; malignant peripheral zone tissue approximates one-tenth

of that level (400–800); and other soft tissues approximate 200–400. Consequently, malignant prostate tissue Zn levels are decreased by ~70–85% compared to normal peripheral zone, and the decrease is observed in the glandular epithelial cells. Most importantly, one rarely, if ever, finds malignant glands that have retained the high Zn levels that characterize the normal gland. In addition, the decrease in Zn occurs early in the development of prostate malignancy [6,7]. These established clinical relationships have raised important issues that relate to the role and mechanisms of Zn accumulation in the normal functioning of the prostate gland and the loss of Zn accumulation as a requirement in the development of prostate malignancy.

It has been shown by Costello and Franklin groups that the functional role of Zn accumulation is to inhibit citrate oxidation of the highly specialized secretory epithelial cells, which permits the production and secretion of unusually high levels of citrate as a major component of prostatic fluid [7,10]. In addition, high Zn levels in the mitochondria inhibits terminal oxidation, truncating the Krebs cycle, hence decreasing the ATP-based energy production and resulting in growth/proliferation and inducing mitochondrial apoptogenesis [12,15]. And this process subsequently inhibits tumor invasion [15]. The combination of such effects can be characterized as anti-tumor effects, which lead us to propose that Zn is a tumor-suppressor agent against prostate cancer. This provides the explanation for the requirement that malignant cells lose the capability to accumulate Zn and the basis for the absence of malignant glands that retain high levels of zinc.

This has led us to pursue the critical issues regarding the mechanism of Zn accumulation in the normal epithelial cells along with the mechanism for the lost ability of the malignant cells to accumulate zinc [7]. The members of the Zip family of Zn transporters have been identified as important Zn transporters for the cellular uptake and accumulation of Zn in mammalian cells. More specifically, we have identified three *hZIPs* (*hZIP1*, 2, and 3) that are down-regulated [29]. However, *hZIP1* has shown to be the most important Zn uptake transporter in prostate cells [6,7,15].

In our present report, by utilizing two different methods: one that can differentiate the relative low versus high amounts of intracellular Zn by utilizing specific Zn binding molecules *in situ* and another one that can differentiate the relative degree of *hZIP1 in situ* by ISRTPCR, we demonstrate that Zn is depleted from the neoplastic as well as pre-neoplastic prostatic glandular epithelial cells. Correspondingly, *hZIP1* is expressed in human normal and hyperplastic prostate glandular epithelium; and is down-regulated in adenocarcinomatous glands. Previously, our group has identified the down-regulation of *hZIP1* expression in the high prostate cancer at-risk African American male population as compared with European American males in a small number of patients we tested [29]. In this report, for the first time, we show down-regulation of *hZIP1* in a much larger group of patients and also show that Zn accumulation is very low in the adenocarcinomatous glands.

4.2. Significance of the methods

In the present studies, we present a new method to observe the relative Zn levels *in situ* in tissues. This method, either alone or in conjunction with *in situ* hybridization method, can be used in many different fields of science including: geomedicine (the science dealing with the influence of natural factors on the geographical distribution of problems in human and veterinary medicine: [41]), nutrition and human health research [42], in phytoremediation [43], toxicology [44], in ecological research [45], in nanotechnology [46], in environmental science to detect and to determine the remediation efforts [43,45,47,48], and in many other situations where differential detection of Zn may be important. When combined with *in situ* hybridization, the researchers can potentially uncover the molecular mechanisms of certain diseases where the relative Zn accumulation may be an important factor in the pathogenesis of many diseases and disorders (i.e. diabetes, and breast cancer) [49,50].

Acknowledgments

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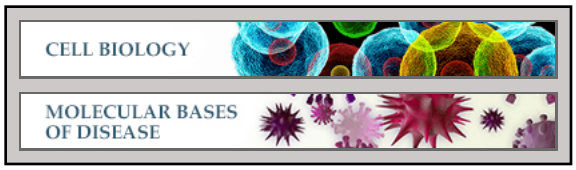
Cell Biology:

miR-183-96-182 Cluster Is Overexpressed in Prostate Tissue and Regulates Zinc Homeostasis in Prostate Cells



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miR-183-96-182 Cluster Is Overexpressed in Prostate Tissue and Regulates Zinc Homeostasis in Prostate Cells*[S]

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Background: Zinc is vital to normal prostate function and uniquely concentrates in healthy prostate. A hallmark of prostate cancers is diminished zinc levels.
Results: The miR-183 family is overexpressed in prostate cancer and regulates intracellular zinc via suppression of zinc transporters.
Conclusion: Prostatic zinc homeostasis is regulated by microRNAs.
Significance: The miR-183 family regulates zinc and may contribute to prostate carcinogenesis.

Decreased zinc levels are a hallmark of prostate cancer tumors as zinc uniquely concentrates in healthy prostate tissue. Increased dietary zinc correlates with decreased risk of advanced prostate cancer and decreased mortality from prostate cancer. The mechanisms of prostatic zinc homeostasis are not known. Lower zinc levels in the tumor are correlated directly with decreased expression of the zinc transporter hZIP1. We report identification of a microRNA cluster that regulates multiple zinc transporters, including hZIP1. Screening in laser capture microdissected prostate cancer tumors identified miR-182 as a potential regulator of hZIP1. Regulation of hZIP1 by miR-182 via two binding sites was confirmed in primary prostate cell cultures. miR-96 and miR-183 are expressed as a cluster with miR-182 and share similar sequences. Array profiling of tissue showed that miR-183, -96, and -182 are higher in prostate cancer tissue compared with normal prostate. Overexpression of the entire miR-183-96-182 cluster suppressed five additional zinc transporters. Overexpression of miR-183, -96, and -182 individually or as a cluster diminished labile zinc pools and reduced zinc uptake, demonstrating this miR cluster as a regulator of zinc homeostasis. We observed regulation of zinc homeostasis by this cluster in prostate cells and HEK-293 cells, suggesting a universal mechanism that is not prostate-specific. To our knowledge, this is the first report of a miR cluster targeting a family of metal transport proteins. Individually or as a cluster, miR-183, -96, and -182 are overexpressed in other cancers too, implicating this miR cluster in carcinogenesis.

Zinc is a vital micronutrient that uniquely concentrates in the healthy prostate at levels 10-fold higher than other soft tissues (1, 2). More than 50 years of research has consistently shown that prostate cancer (PC)[3] lesions have zinc levels ~6-fold lower than adjacent normal prostate (2, 3), suggesting that zinc has a protective role against cancer development (4). The zinc concentration lowers with the Gleason score of the tumor and is specific to PC (5). Benign diseases of the prostate, prostatitis and benign prostatic hyperplasia, maintain normal zinc levels (1, 5). In the TRAMP (transgenic adenocarcinoma of the mouse prostate) model of prostate cancer, fluorescent imaging of zinc in live animals correctly detected tumors at an early stage by low zinc compared with surrounding normal prostate (6). Epidemiological studies have implicated zinc in PC risk and outcome showing that high dietary zinc was associated with a decrease risk of advanced disease (7) and lower PC-specific mortality (8). Thus, the zinc disparity in PC is hypothesized to contribute to PC etiology (7) and is a viable selective biomarker for PC (6).

Low zinc levels in PC are associated with lower expression of the zinc transporter hZIP1 (3, 9). Also known as SLC39A1, hZIP1 is named after its yeast homolog, Zrt-Irt-like protein. There are 14 known human hZIP transporters that act to increase intracellular labile zinc pools by transporting the ion into cytoplasm from either the extracellular space or organelle (10). There are also known 11 human hZnT human zinc transporter, SiBOA1 proteins that function to decrease labile zinc pools by exporting zinc out of the cell or sequestering it into intracellular compartments (10). The mechanism of reduced hZIP1 expression in PC has not been determined.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.
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[3] The abbreviations used are: PC, prostate cancer; miR, microRNA(s); LCM, laser capture microdissection; qPCR, quantitative PCR; luc, luciferase; PrE, primary prostatic epithelial; PrS, primary prostatic stromal; hZnT, human zinc transporter.

MicroRNAs (miR) have emerged rapidly as an important class of short endogenous RNAs that act as post-transcriptional inhibiters of protein expression by base pairing with the 3′-UTR of their target mRNAs. As miR profiling studies have become widespread, aberrant levels of specific miR have been observed in most cancers. miR profiles in PC have been reported (reviewed in Ref. 11), but the miR deregulated in PC have little overlap between studies, which is likely due to differences in study design, sample collection methods, heterogeneity of contaminating stromal cells, and sensitivity/specificity of the detection platforms (11).

We hypothesized that lower levels of the hZIP1 protein and zinc in PC are due to aberrant expression of one or more miR. miR complementary to hZIP1–3′-UTR were screened in laser capture-microdissected prostate epithelium from PC patients. Putative zinc-regulating miR were further investigated *in vitro* using human primary prostate cultures and PC cell lines. We report the ability of specific miR to regulate intracellular zinc via regulation of zinc transporters.

EXPERIMENTAL PROCEDURES

hZIP1 and miRNA Expression in Patient Tissues—Radical prostatectomy specimens from 10 male patients (five Caucasians and five African-Americans) were selected for analysis via an Institutional Review Board-approved protocol at the University of Illinois Medical Center at Chicago. Normal and PC epithelium was collected from 8-μm Formalin-Fixed paraffin-embedded prostate sections by laser capture microdissection (LCM) with Lieca LMD-100 as described previously (12). Total RNA was isolated using RecoverAll (Invitrogen). Reverse transcription and quantitative PCR (RT-qPCR) with TaqMan Assays for GAPDH, HPRT1, hZIP1, RNU44, RNU48, miR-100, miR-96, miR-30c, miR-223, miR-346, and miR-182 was performed as detailed below and described previously (12).

Cell Cultures—Primary prostatic epithelial (PrE) and stromal (PrS) cells were established from histologically normal areas of the prostate peripheral zone from patients undergoing radical prostatectomy via an Institutional Review Board-approved protocol (University of Illinois Medical Center at Chicago) and maintained as described previously (13, 14). PrE cells were maintained on collagen-coated plates, grown in serum-free prostate epithelial growth medium (Lonza, Walkersville, MD) and used on secondary passage and at ~75% cell density. PrS cells were maintained in MCDB-105 (Sigma) supplemented with 10% fetal bovine serum. LNCaP, PC3, and HEK-293 cell lines were obtained from ATCC (Rockville, MD) and maintained as directed. To control for density-dependent fluctuations in miR expression (15), miR were measured in all the cells at 75% confluence 24 h after a medium change.

RT-qPCR—For mRNA analysis, cDNA was generated from total RNA (50 ng for LCM-collected RNA, 500 ng for cell cultures) using Vilo Reverse Transcriptase (Invitrogen). For miRNA assays, stem-loop RT was carried out on 10 ng of total RNA with an assay-specific primer using TaqMan miRNA RT Kit (Invitrogen). qPCR was run on Step One Plus (Applied Biosystems) with miRNA or mRNA-specific TaqMan assays. Results were normalized to housekeeping RNAs by ΔC_t method (16).

Immunoblot for hZIP1 Protein—Cells were collected into protein lysis buffer (Cell Sigaling, Danvers, MA), sonicated, and centrifuged to remove insoluble fraction. Protein (25 μg) was run on a 10% Bis-Tris NuPAGE gel and transferred to PVDF membrane. After a 1-h block at room temperature in TBS/0.1% Tween/5% milk, chicken polyclonal anti-hZIP1 (generous gift from Dr. Renty Franklin at the University of Maryland, Baltimore, MD) was probed at 1:10,000 and anti-β-tubulin (Cell Signaling, Danvers, MA) was probed at 1:2000 overnight at 4 °C. Secondary HRP-conjugated anti-chicken or anti-rabbit was used 1:1000 at room temperature for 1 h. hZIP1 protein levels were quantified as a ratio to β-tubulin using ImageJ software (17).

Pre-miR Transfection—Negative control scrambled pre-miR (NEG) or pre-miR to miR-183, miR-96, or miR-182 (Invitrogen) were transfected into cells at specified concentration (5–50 nM) by reverse transfection using siPORT NeoFX (Invitrogen) according to the manufacturer's instructions.

Luciferase Assay—Cells were seeded into 24-well plates and used at 75% confluence. hZIP1–3′-UTR or random 3′-UTR 2 (Switchgear Genomics, Menlo Park, CA), pRL-null (Promega, Madison, WI), and pre-miR (Invitrogen) were co-transfected into cells using Dharmafect Duo (Dharmacon). Dual-Luciferase assay (Promega) was run 24 h after transfection. Data shown as ratio of luc/*Renilla*-luc to normalize for transfection efficiency. Mutations were introduced to the two predicted miR-182/96 binding sites of the hZIP1 3′-UTR reporter construct (mutagenesis primers, 5′-ccagtcctgcttactcctctatttttaaagggacg-agcaaatccccttcctctttctc-3′ and 5′-catgcccagatgataaacactgagcg-gacgagacatttttttaaatacacccgagga-3′) following the recommended protocol for the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenized construct was confirmed by sequencing.

miR-183-96-182 Expression in Outside Patient Data Set—The expression of the miR-183-96-182 cluster was assessed in an outside patient data set from the tissue bank of the Erasmus University Medical Center (Rotterdam, the Netherlands). 50 fresh-frozen primary prostate cancer and 11 normal adjacent prostate samples were obtained by radical prostatectomy. Histological evaluation and Gleason grading were performed by two pathologists on hematoxylin/eosin-stained frozen sections for all analyzed samples. Tumor samples in which at least 60% of the cells were cancerous and contained at least 60% ducts were selected for analysis. The non-tumor samples contained 0% tumor cells and at least 60% ducts. Collection of patient samples has been performed according to the Dutch national legislation concerning ethical requirements. Use of these samples has been approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004-261).

Total RNA was isolated from frozen tissue samples using RNABee reagent (Campro Scientific, GmbH) according to the manufacturer's protocol. Microarray analysis of miRNA expression was performed using human miR V2 microarrays (Agilent) that contain probe sets for 723 human microRNAs from the Sanger MiRBase (version 10.1). Microarray pre-processing and hybridization were performed according to the manufacturer's protocols. Prior to analysis, raw hybridization

TABLE 1
miRNAs and hZIP1 expression in patient prostate tissue
$p < 0.05$, indicated in bold.

mRNA	Correlation with hZIP1[a]	Fold change in PC[b]
miR-30c		
All patients	$r = -0.04$ ($p = 0.86$)	**-2.39 ($p = 0.01$)**
Cauc patients	$r = -0.38$ ($p = 0.27$)	-3.03 ($p = 0.10$)
AA patients	$r = 0.34$ ($p = 0.33$)	**-2.21 ($p = 0.03$)**
miR-100		
All patients	$r = -0.12$ ($p = 0.62$)	-2.01 ($p = 0.06$)
Cauc patients	$r = -0.26$ ($p = 0.46$)	-1.59 ($p = 0.27$)
AA patients	$r = 0.16$ ($p = 0.64$)	-2.40 ($p = 0.07$)
miR-182		
All patients	$r = -0.33$ ($p = 0.10$)	1.43 ($p = 0.24$)
Cauc patients	**$r = -0.77$ ($p = 0.01$)**	2.37 ($p = 0.14$)
AA patients	$r = 0.29$ ($p = 0.39$)	1.34 ($p = 0.34$)
miR-223		
All patients	$r = -0.14$ ($p = 0.54$)	**-2.23 ($p = 0.04$)**
Cauc patients	$r = -0.53$ ($p = 0.12$)	-3.32 ($p = 0.16$)
AA patients	$r = 0.38$ ($p = 0.27$)	-1.77 (0.07)
miR-346		
All patients	$r = -0.16$ ($p = 0.48$)	-2.26 ($p = 0.06$)
Cauc patients	**$r = -0.69$ ($p = 0.03$)**	-3.01 ($p = 0.07$)
AA patients	$r = 0.34$ ($p = 0.32$)	-1.15 ($p = 0.31$)
hZIP1		
All patients	NA[c]	**-1.29 ($p = 0.05$)**
Cauc patients	NA	-1.52 ($p = 0.11$)
AA patients	NA	-1.20 ($p = 0.19$)

[a] Spearman's correlation coefficient between hZIP1 mRNA and miRNA, p value based on $n = 20$ observations; normal and PC samples from 10 patients (five Caucasians (Cauc) and five African-Americans (AA)).
[b] Average fold change in PC compared to normal tissue with p value from paired student's t-test from $n = 26$ observations; normal and PC samples from 10 patients (five Caucasians and five African-Americans).
[c] NA, not applicable.

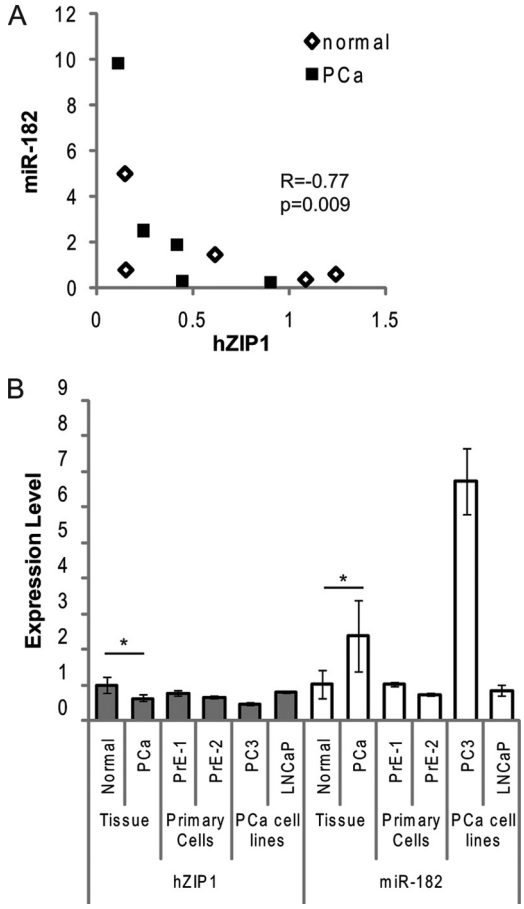

FIGURE 1. **hZIP1 and miR-182 levels in patient tissues and in primary human prostate epithelial cells.** *A*, Spearman correlation between miR-182 and hZIP1 mRNA in LCM-collected patient prostate epithelial tissue samples measured by RT-qPCR. Relative levels of expression are on the axes, and the Spearman ρ and p value are shown ($n = 10$; five patients, normal (*open diamonds*) and PC (*squares*) tissue for each). Data shown are relative value to patient 1. *B*, RT-qPCR analysis of basal levels of hZIP1 mRNA and miR-182 in LCM-collected epithelium from PC (*PCa*) patients ($n = 5$), primary normal epithelial cells (PrE-1 and PrE-2, cells from two patients), and PC cell lines (PC3 and LNCaP). Data are shown relative to PrE-1. miR-182 normalized to RNU44 and hZIP1 normalized to GAPDH. *, $p < 0.01$ paired *t* test between the normal and cancer tissue. The graph is representative of three independent experiments, and *error bars* represent S.D. of technical duplicates.

signals were background-subtracted, quantile-normalized, and scaled to the median of all samples. miR that were assigned a present call in at least 50% in any one of the tested conditions were examined for differential expression by a Welch t test (unpaired, two tails, α-level, 0.05), followed by multiple testing correction by the method of Benjamini and Hochberg (corrected p value cut-off of 0.05) (18) miR that passed the t test and had significant corrected p values were further filtered on difference in fold change between both conditions (cut-off > 1.5). The array data were submitted using Tab2MAGE to the ArrayExpress database (accession no. E-TABM-794).

Intracellular Zinc Concentration—Cells were reverse-transfected with pre-miR (50 nM) via NeoFX siPORT into 30-mm collagen-coated dishes per the manufacturer's instuctions. Following transfection (48 h) cells were scraped into EDTA-free cell lysis buffer and lysed by freeze-thaw and sonication. Protein was quantified by Bio-Rad protein reagent (Bio-Rad), and 50 μl were loaded onto a QuantiChrom™ zinc assay kit (BioAssay Systems, Hayward, CA) and visualized according to manufacturer's protocol. Results are shown as pmol of zinc per μg of protein.

Zinc Import—HEK-293 cells (7500 cells/well) were reverse-transfected with pre-miR as described above in collagen-coated 96-well clear-bottomed black-walled plates. 48 h after transfection, cells were loaded with 2 μM FluoZin-3 (Invitrogen) for 30 min. Cells were washed twice with medium and allowed to equilibrate for 15 min. Medium was changed to phenol red-free HBS (HEPES-buffered saline) for fluorescence. $ZnSO_4$ (final concentration, 150 μM) was added, and fluorescence data (excitation, 494 nm; emission, 516 nm) were collected in every 30 s for 10 min (Synergy HT, BioTek, Winooski, VT). Following subtraction of background fluorescence (without zinc addition), fluorescence at 516 nm was analyzed as percentage of time zero fluorescence. Representative image of FluoZin-3 fluorescence counterstained with Hoechst 33342 with and without zinc was collected with the Zeiss LSM 510 confocal microscope.

RESULTS

miR-182 Expression Inversely Correlated with hZIP1 in PC Patient Tissue—We rationally selected six miR (miR-30c, miR-96, miR-100, miR-182, miR-223, and miR-346) for screening as putative regulators of hZIP1. Because hZIP1 levels are lower in PC, miR selection was based on computationally predicted binding to hZIP1–3′-UTR (TargetScan,version 5.1 (19), miRanda-mirSVR (20), Human MicroRNA Targets by Memorial Sloan-Kettering Cancer Center Computational Biology (21)), and/or reported overexpression in PC (22). Expression of

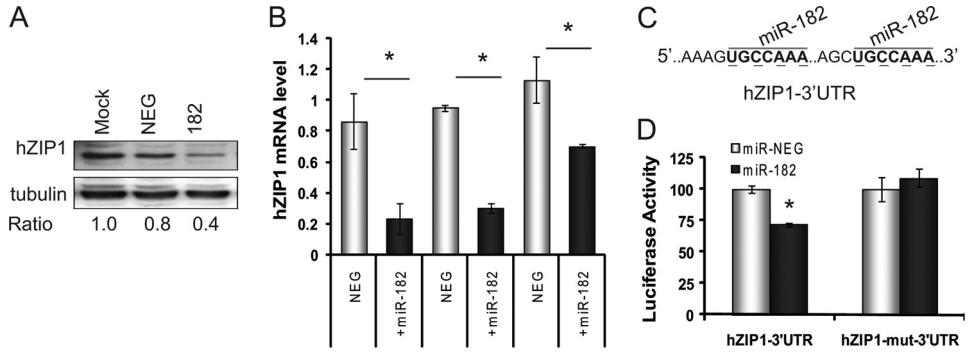

FIGURE 2. **Regulation of hZIP1 by miR-182 and validation of miR-182 binding sites in hZIP1 3′-UTR in normal PrE cells.** *A*, immunoblot of hZIP1 protein in PrE cells 48 h following transfection with 50 nmol of pre-miR-182 or pre-miR-NEG (ratio to β-tubulin). *B*, hZIP1 mRNA in two PrE cell lines and in LNCaP prostate cancer cells 48 h following transfection with 50 nmol of pre-miR-182 or pre-miR-NEG. Results are shown relative value to pre-miR-NEG transfection. hZIP1 normalized to β_2-microglobulin. *Error bars* represent S.D. of replicate experiments. *C*, predicted miR-182 binding sites in hZIP1 3′-UTR and site-directed mutagenesis locations (*underlined in boldface type*). *D*, luciferase activity of luc-hZIP1–3′-UTR or luc-hZIP1-mut-3′-UTR 24 h following co-transfection with prL-null, pre-miR-182 or pre-miR-NEG. Results were normalized to *Renilla* luciferase and shown as percentage of control. *Error bars* represent S.D. of replicate experiments. *, $p < 0.05$ paired *t* test. Results are representative of three or more independent experiments.

these miR and hZIP1 was measured in LCM-collected PC and adjacent histologically normal epithelium from 10 radical prostatectomy specimens. Because hZIP1 levels were shown previously to be lower in African-American men (9), we performed analysis on the specimens as a whole and by race. In our small specimen set, we did not observe a statistically significant difference in the expression levels for hZIP1 mRNA for any of the miRNAs between the African-American ($n = 5$) and Caucasian men ($n = 5$) (data not shown). Both miR-182 and miR-346 expression inversely correlated with hZIP1 mRNA (Spearman $\rho = -0.77$, $p = 0.03$; and $\rho = -0.69$, $p = 0.03$, respectively) (Table 1 and Fig. 1*A*) in Caucasians only. miR-182 tended to be higher in PC tissue (*t* test $p = 0.18$, $n = 10$) but was not statistically significant in our small data set. Interestingly, miR-30c, miR-346, miR-223, and miR-100 were present at lower levels in PC tissue (Table 1), consistent with previous reports of global miR down-regulation in PC (23). miR-96 was not detectable in the patient samples.

miR-182 Regulates hZIP1 Expression in Vitro—miR-182 was selected for *in vitro* studies because it was expressed higher in PC and had two putative binding sites in the hZIP1–3′-UTR, as opposed to miR-346, which was lower in PC and only had one binding site in hZIP1. We examined hZIP1 and miR-182 expression in two prostate cancer cell lines (LNCaP, PC3), in two patient-derived primary prostatic epithelial cell cultures (PrE-1, PrE-2) and in primary prostatic stromal cells (PrS-1, PrS-2). Both hZIP1 and miR-182 were expressed specifically in epithelial-derived primary cultures and PC cell lines (Fig. 1*B*). The PC cell line PC3 showed the highest level of miR-182 and the lowest of hZIP1 mRNA. Neither hZIP1 nor miR-182 was expressed highly in PrS cells (Fig. 1*B*), which is consistent with zinc accumulating in zinc epithelium.

We next tested the ability of miR-182 to alter hZIP1 in cells. Transient transfection of pre-miR-182 decreased hZIP1 protein in PrE cells (Fig. 2*A*). Pre-miR-182 also decreased hZIP1 mRNA in PrE cell lines (data from two patients PrE-1 and PrE-2) and in LNCaP cells (Fig. 2*B*). Specific overexpression of the mature miR-182 following pre-miR transfection was confirmed by RT-qPCR (supplemental Fig. S1). The 3′-UTR of hZIP1 (NM_014437.3) contains two putative binding sites for miR-182 at positions 574 and 814 (Fig. 2*C*). Transfection of cells with pre-miR-182 decreased luciferase activity of a luc-hZIP1–3′-UTR construct (Fig. 2*D*). To confirm the putative miR-182 binding sites, the sites were mutated by site-directed mutagenesis (Fig. 2*C*). miR-182 transfection did not alter luciferase activity of the hZIP1–3′-UTR-mut construct (Fig. 2*D*). These experiments confirmed regulation of hZIP1 by miR-182 via two binding sites in the 3′-UTR.

Expression of miR-183, miR-96, and miR-182 Cluster Is Higher in PC—miR-182 is transcribed on a polycistronic RNA strand with miR-96 and miR-183 (Fig. 3*A*), which is then processed into the three mature miR (24). All three miR have a high degree of sequence homology and potentially overlapping mRNA targets (Fig. 3*A*). Therefore, we investigated the expression of miR-183 and miR-96 in prostate tissue and cells and the ability of these miR to regulate hZIP1.

miR microarray profiling of an outside patient cohort of 50 patients with organ-confined prostate tumors and 11 normal controls demonstrated that the expression levels of miR-183, miR-96, and miR-182 were increased with at least 2-fold in PC tissue compared with normal adjacent prostate (Fig. 3*B*) (25). The observed up-regulation was found significant when examined by a Welch *t* test, followed by multiple testing correction by the method of Benjamini and Hochberg (corrected *p* values of 0.002, 0.0002, and 0.00008, for miR-183, miR-96, and miR-182, respectively). Correlation analysis of the miR showed that expression levels of miR-183 and miR-96 are highly correlated to each other in patients ($r = 0.77$, $p < 0.0005$) but that miR-182 levels did not correlate with the levels of other two miR (supplemental Table S1). Zinc transporter levels for these patients were not included in this data set. These results indicate that in addition to miR-182, miR-183, and miR-96 are present at higher levels in PC and may contribute to hZIP1 mRNA regulation. Also, the lack of expression correlation between all three miR suggest that there is secondary regulation of the individual miR following transcription as a single transcript as miR-183-96-182.

miR-183 was not included in our original miRNA screen in patients, and miR-96 was undetectable by our technique. All three miR (183, 96, and 182) are present in prostatic epithelial

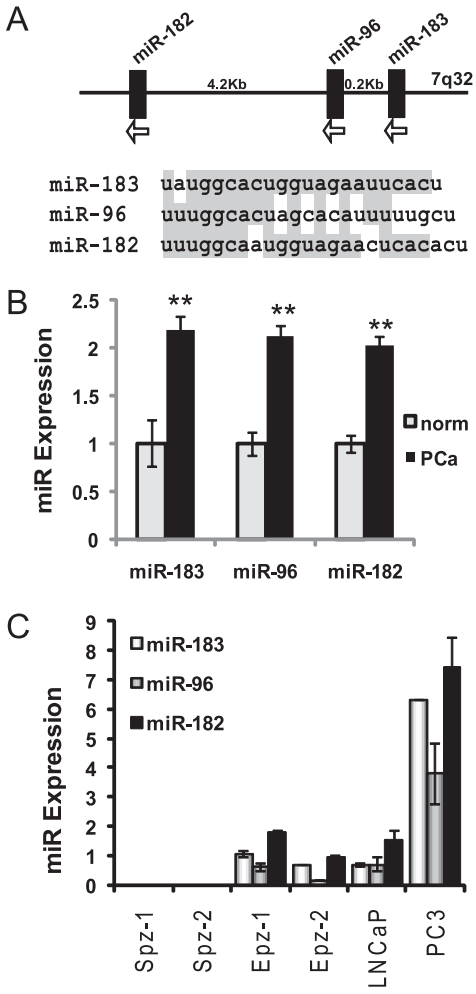

FIGURE 3. **miR-183-96-182 cluster expression in prostate cells and patient tissue.** *A*, miR-183-96-182 expressed as polycistronic RNA on chromosome 7 and sequence alignment for human miR-183, miR-96, and miR-182. *B*, miR-183, -96, and -182 expression in human prostate tissue by cDNA array. Graphs shows mean expression ± 95% confidence intervals for normal (*norm*) prostate ($n = 11$) and PC (*PCa*) tissue ($n = 50$). **, $p < 10E\text{-}7$ unpaired *t* test. Adaptation of data was by Martens-Uzunova *et al.* (25). *C*, basal levels of miR-183, miR-96, and miR-182 in human prostate cell cultures by RT-qPCR. Results shown relative to PrE-1 and normalized to RNU44 and RNU48. *Error bars* represent S.D. of duplicate experiments.

PrE and PC cell lines, but not in prostatic stromal PrS cells (Fig. 3*C*). miR-96 was lower in all cells that expressed the cluster, suggesting expression regulation secondary to transcription. PC3 cells, which are androgen-independent, express higher levels of the cluster, suggesting that the expression of miR-183-96-182 may further increase with disease progression.

miR-183, -96, and -182 Regulate hZIP1—Because miR-183, -96, and -182 are expressed biologically together as a cluster, we tested the ability of the miR to regulate hZIP1 individually and as a cluster. Transient transfection with pre-miR showed strong down-regulation of hZIP1 mRNA and protein by miR-96 and miR-182 (Fig. 4, *A* and *B*), which is consistent with the predicted binding sites for those miR. miR-183 lowered hZIP1 mRNA to a lesser degree, but the inhibitory effect on hZIP1 was strongest when all three miR were expressed in concert. Interestingly, although miR-182 and -183 have higher homology overall, miR-183 has a one-base difference near the 5′ end where hZIP1 binding occurs (Fig. 3*B*). Our results suggest that this small change is enough to alter its affinity for the target.

The two putative bindings sites for miR-182 in hZIP1 3′-UTR are highly conserved across species (supplemental Fig. S2). Despite highly similar sequences, target prediction software predicted the formation of hybrids between hZIP1 and miR-96 and -182, not with miR-183. Alignment and free energy of hybridization were calculated for each of the miR with hZIP1 using RNAhybrid (45) (supplemental Fig. S3).

miR-183, -96, and -182 all inhibited the luc-hZIP1–3′-UTR construct and had no effect on the mutated 3′-UTR (Fig. 4*C*), indicating functionality of the putative miR binding sites. Mutation of the miR binding sites individually showed that miR-96 preferentially binds to the 575 site, whereas miR-182 can bind both of the sites (Fig. 4*C*).

miR-96 and miR-182 Inhibit Zinc Uptake—To determine whether altering hZIP1 levels was of biological significance, we examined intracellular zinc concentration. Overexpression of miR-96 and miR-182 and miR-183-96-182 together significantly lowered intracellular zinc levels in PrE cells (Fig. 5*A*). miR-183 alone lowered zinc to a lesser degree. These results show that the cluster of miR-183-96-182 inhibits hZIP1.

We next examined intracellular zinc import. HEK-293 (human embryonic kidney) cells were used to measure zinc uptake as these cells transfect at high efficiency and have been used previously for zinc transport assays (26). In the HEK-293 cells, miR-183, -96, and -182 regulate hZIP1 expression and luc-hZIP1–3′-UTR similarly to our previous observations in prostate cells (Fig. 5*A*). Two days after transfection with pre-miR, cells were loaded with FluoZin-3, a fluorescent dye that specifically binds to intracellular labile zinc (Fig. 5*B*). Then, zinc was added to the cells and the intracellular zinc content measured over time via FluoZin-3 fluorescence. Because zinc was added in excess to the medium, this method will show the overall zinc uptake into the cell. The results demonstrate the amount of zinc in the cell at each time point but do not differentiate between the rate of zinc import and export. Regulation of zinc uptake in HEK-293 cells echoed our previous results for total intracellular zinc in that expression miR-96, miR-182, or all three miR inhibited zinc uptake, whereas miR-183 alone only trended to decrease the zinc (Fig. 5*C*). These results show that acute intracellular zinc import is attenuated by miR-96/182. Also, as these experiments were carried out in HEK-293 cells, which are human embryonic kidney cells, demonstrates that zinc regulation by the miR is not unique to prostate cells.

miR-183-96-182 Regulate Multiple Zinc Transporters—Given the marked effect of the miR on zinc levels and the presence of other zinc transporters in prostate cells (27), we next examined the ability of this miR cluster to regulate other zinc transporters. We selected additional zinc transporters based on computational predicted miR target analysis and on a study by Xu *et al.* (24), which showed five hZIPs and three hZnTs are putative mRNA targets for miR-183, miR-96, or miR-182. Of the eight additional zinc transporters analyzed, overexpression of miR-183-96-182 together in PrE cells suppressed mRNA levels of six zinc transporters (hZIP1, hZIP3, hZIP7, hZIP9, hZnT1, and hZnT7) and did not affect two zinc transporters (hZIP10, hZnT9) (Table 2). Transfection with the miR individ-

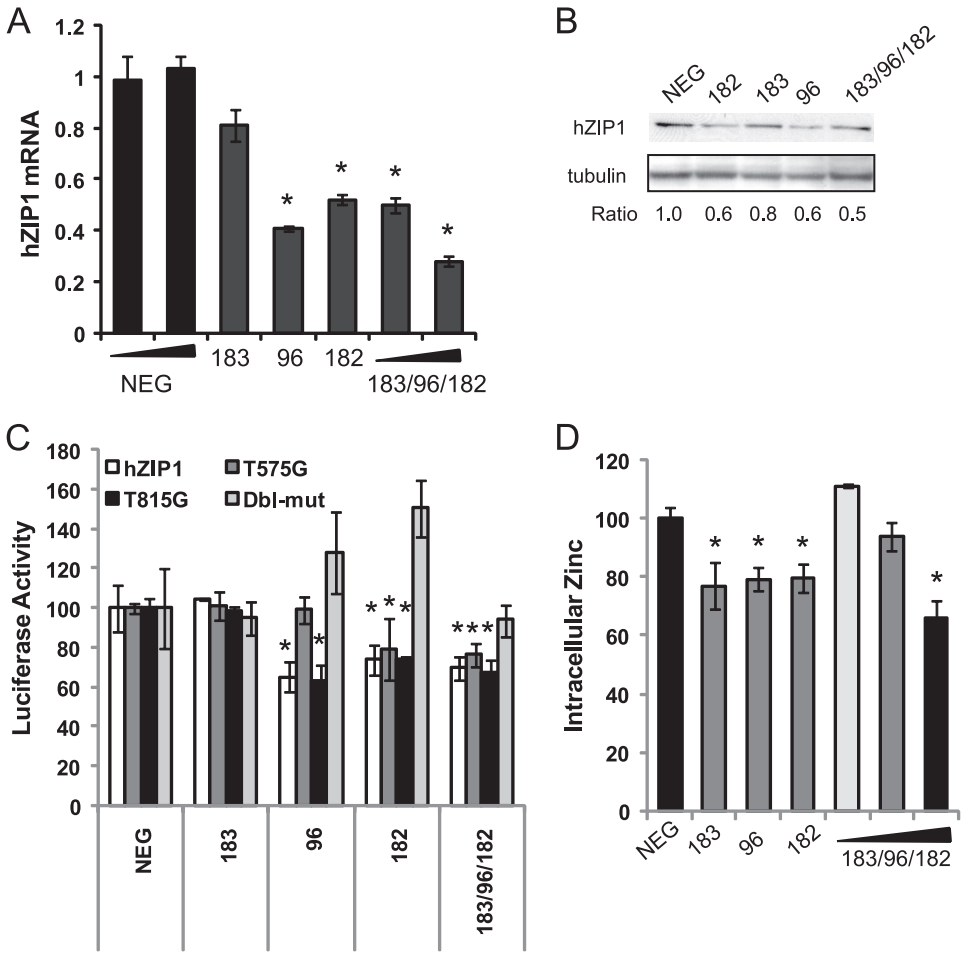

FIGURE 4. **miR-183-96-182 cluster regulation of hZIP1 and intracellular zinc in primary prostate epithelial cells.** hZIP1 mRNA (*A*) and protein (*B*) levels 24 h after transfection of miR-183, miR-96, or miR-182 in PrE cells. mRNA measured by RT-qPCR and protein by immunoblotting. Ratio to β-tubulin for immunoblot. *C*, luciferase activity of luc-hZIP1–3′-UTR or luc-hZIP1-mut-3′-UTR 24 h following co-transfection with prL-null, pre-miR-182 or pre-miR-NEG. Results were normalized to *Renilla* luciferase and shown as percentage of control. *Error bars* represent S.D. of replicate experiments. *D*, intracellular zinc in PrE cells 48 h following transfection with 15–50 nM pre-miR to miR-183, miR-96, and miR-182. *Error bars* are S.E. of three experiments. *, $p < 0.05$ paired *t* test.

ually suppressed zinc transporter mRNAs as predicted by binding sites in the 3′-UTRs, but the effects were stronger when the miR were transfected together. FOXO3 has previously been reported to be regulated by miR-182 and miR-96 (28, 29) and was included as a positive control.

DISCUSSION

Intracellular zinc is tightly controlled as zinc is required for many physiological processes, including structural functions, catalytic functions, gene expression, protein-protein interactions, fatty acid metabolism, apoptosis, and signal transduction (30). Unlike most organs that maintain very low zinc levels, the prostate distinctively concentrates zinc, a phenotype that is diminished in malignant prostate. In PC tissue, we observed consistently lower levels of the zinc transporter, hZIP1, and screened for miR inversely correlated to hZIP1. We identified miR-182 as being inversely associated with hZIP1 mRNA in PC patients. A caveat to this approach is that any miR overexpressed in PC would inversely correlate with hZIP1 but not necessarily be a regulator of hZIP1. Therefore, we made use of cell cultures to validate hZIP1 as a *bona fide* target of miR-182. Not only did we confirm miR-182 as a regulator of hZIP1, we observed regulation of other zinc transporters and labile zinc by miR-183, -96, and -182. During preparation of this work, Zou *et al.* (31) showed that RREB-1 (ras responsive element binding protein-1) contributes to regulation of hZIP1 in the PC3 cell line. In agreement with their data, in our experiments, hZIP1 expression was not altered in PC3 cells transfected with miR -183, -96, and -182 (data not shown). The RREB-1 regulation of hZIP1 may also fit into the miR-183 family regulation of zinc as RREB-1 contains a putative binding sites for miR-183. Although not included in our study, regulation of zinc homeostasis by MTF-1 (metal-responsive element binding transcription factor-1), metallothionein proteins, and others should not be discounted.

Clustered miRNAs account for 37% of human miRNAs and are thought to exist as a mechanism to more efficiently coordinate complex cell processes than regulation by a single miRNA can provide (32). Mature miR-183, -96, and -182 have similar sequences and are highly conserved across species (humans to zebrafish), suggesting that they are the result of an ancient gene duplication. We show here that miR-183, -96, and -182 function to target several proteins involved in zinc import/export to have an overall regulation of zinc homeostasis. We observed

regulation of zinc homeostasis by this cluster in both prostate cells and HEK-293 cells, suggesting a universal mechanism that is not unique to the prostate. The mechanism that regulates expression of this cluster is not known. miR-182 expression has been shown to be responsive to ionizing radiation (33). Of note, we did not observe changes in miR-183-96-182 following exposure to high levels of zinc (data not shown).

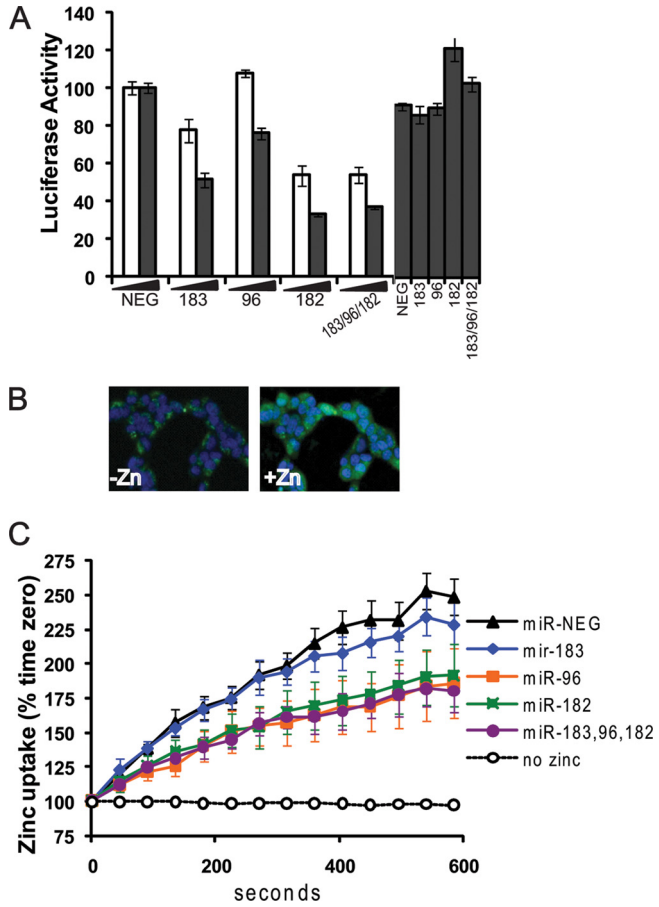

FIGURE 5. **miR-183-96-182 cluster regulates intracellular zinc uptake.** *A*, luciferase activity of luc-hZIP1–3′-UTR or luc-hZIP1-mut-3′-UTR 24 h following co-transfection with prL-null, pre-miR-183, pre-miR-96, pre-miR-182, or pre-miR-NEG (nM of pre-miR indicated on *x* axis). Results were normalized to *Renilla* luciferase and shown as percentage of control. *Error bars* represent S.D. of replicate experiments. *, $p < 0.05$ paired *t* test. *B*, intracellular zinc in HEK-293 cells imaged with FluoZin-3 (*green*) and nuclei stained with Hoescht (*blue*) 0 and 3 min after adding 150 μM ZnSO4. *C*, quantification of zinc uptake by FluoZin-3 fluorescence in HEK-293 cells. Data collected every 45 s for 10 min and shown as percentage of time zero fluorescence. Zinc uptake measured 24 h following transfection with 50 nM pre-miR-NEG, pre-miR-183, pre-miR-96, or pre-miR-182. The *no zinc line* is a negative control to demonstrate no change in FluoZin-3 fluorescence in the absence of zinc. Data points represent the mean, and *error bars* represent S.E. of three independent experiments that each contained technical duplicates. *, $p < 0.05$ paired *t* test.

Either individually or as a cluster, the levels of miR-183, miR-96, and miR-182 have been shown to be deregulated in cancer, autoimmunity, and senescence. The expression of miR-183-96-182 cluster is higher in PC compared with normal prostate in a recent study by Schaefer *et al.* (34). They further found that expression of these miR along with miR-149, -181b, -205, and -375 correctly identified PC from normal tissue with an area under the curve of 0.88 (34). miR-96 and miR-182 are expressed at a high level in urothelial carcinoma and detection of the miR in urine correlated with disease stage and grade (35). Dai *et al.* (36) recently reported a 10-fold increase in miR-183-96-182 cluster expression in splenocytes in a mouse model of lupus, suggesting a role for this cluster in autoimmunity. miR-183 and miR-182 were overexpressed in stress-induced premature senescence *in vitro* (37). Of relevance to zinc, the miR-183-96-182 cluster is highly expressed in the adult mammalian retina (24), also a zinc-rich organ like prostate, and may be involved in insulin signaling (38).

Mature miR-183, -96, and -182 have sequence similarity but have distinct and common mRNA targets. Both miR-96 and miR-182 are reported to target FOXO1 and FOXO3 in breast and melanoma cancer cell lines and endometrial tumors (28, 29, 39; Myatt, 47). mir-182 also targets BRCA1 and may sensitize cells to poly(ADP-ribose) polymerase inhibitors (33). The miR-182 knock-out mouse does not have an apparent phenotype (40), suggesting potential compensation by miR-96 and miR-183. Of note, we analyzed prostate tissue from these mice and did not detect a difference in hZIP1 in the miR-182$^{-/-}$ mice (data not shown), further suggesting that there is redundancy in the targets of the miR-183-96-182 cluster. miR-183 has mRNA targets distinct from miR-96 and -182 despite similar sequences. Sarver *et al.* (41) showed that miR-183 was highly expressed in synovial sarcomas and acts as an oncomir by targeting EGR1 mRNA.

The questions remain on whether dietary zinc is chemoprotective in the prostate or whether low zinc in PC is tied to its dedifferentiated phenotype. Animal and epidemiology studies have shown mixed results for the chemopreventive activity of dietary zinc. In the TRAMP mouse model of PC, dietary zinc supplementation reduced tumor size and PC serum indicators,

TABLE 2
Regulation of zinc transporters by miR-183-96-182 cluster

Zinc transporter	miR sites in 3′-UTR (no.)	Change in mRNA by miR overexpression[a]			
		miR-183	miR-96	miR-182	miR-183/96/182
		%			
hZIP1	miR-182 (2), miR-96 (2)	−25	−72	−73	−76
hZIP3	miR-96 (1)	−24	−41	−24	−34
hZIP7	miR-182 (1), miR-96 (1)	−20	−43	−30	−27
hZIP9	miR-96 (1)	+18	−23	−26	−45
hZIP10	miR-96 (1)	+45	−2	−7	−8
hZnT1	miR-182 (1)	+57	+27	−37	−15
hZnT7	miR-182 (1)	−17	−33	−50	−55
hZnT9	miR-183 (1)	−28	+1	−1	−15
FOXO3	miR-182 (2), miR-96 (2)	−25	−34	−15	−33

[a] PrE cells transfected for 48 h with 50 nM of pre-miR to -183, -96, or -182. mRNA quantified by qRT-PCR were normalized to TATA box-binding protein and shown as percent change compared to cells transfected with pre-miR-neg (scrambled). Shown are the mean of three independent experiments with three different PrE cell lines.

supporting a chemoprotective effect of zinc (42). There are two large cohort studies that looked at supplemental zinc and PC risk. Gonzalez *et al.* (7) found that zinc supplementation did not decrease in overall PC but did decrease the risk of advanced PC diagnosis (hazard ratio = 0.34). In a Swedish cohort, there was no association between dietary zinc and PC (43), but high zinc supplementation did associate with reduced risk of PC-specific death (hazard ratio = 0.64) (8).

There are several hypotheses on the role of zinc disparity in prostate carcinogenesis. One is that low zinc changes the metabolic state of the prostate cell and is thus a contributor to carcinogenesis. The proposed mechanisms is that high zinc in normal prostate inhibits mitochondria aconitase, causing decreased citrate oxidase (44). In contrast, the lower zinc in PC may facilitate increased citrate oxidation, which then increases available energy that is required for tumor growth. Another hypothesis is that high zinc is a phenotype of differentiated luminal prostate cells, therefore PC cells, which are in dedifferentiated state, have lost the ability to sequester zinc because of dedifferentiation. Therefore, although low zinc is a robust phenotype of PC, it is not clear whether low zinc contributes to carcinogenesis or is a result of carcinogenesis.

In summary, we show that the cluster of miR-183-96-182 is a regulator of intracellular zinc concentrations in prostate cells. Furthermore, we and others have shown overexpression of this miR cluster in PC, implicating a role for this miR-cluster in carcinogenesis. Further analysis of miR-183-96-182 in preneoplastic lesions is needed to investigate its regulation in early disease. Mouse transgenic and knock-out models will provide insight into the role of this cluster in the development of normal prostate, zinc homeostasis, and prostate carcinogenesis.

Zinc is an essential micronutrient and the discovery of the control of intracellular zinc by a highly conserved miR cluster is novel and suggests an epigenetic component to zinc homeostasis within cells. This finding has distinctive relevance to PC given the marked decrease in zinc levels and its potential in promoting this disease. Finally, our results have implications not only in PC but also in other cancers and in other organs with high zinc.

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Prostate Cancer Outcome and Tissue Levels of Metal Ions

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BACKGROUND. There are several studies examining prostate cancer and exposure to cadmium, iron, selenium, and zinc. Less data are available on the possible influence of these metal ions on prostate cancer outcome. This study measured levels of these ions in prostatectomy samples in order to examine possible associations between metal concentrations and disease outcome.

METHODS. We obtained formalin fixed paraffin embedded tissue blocks of prostatectomy samples of 40 patients with PSA recurrence, matched 1:1 (for year of surgery, race, age, Gleason grading, and pathology TNM classification) with tissue blocks from 40 patients without recurrence (n = 80). Case–control pairs were compared for the levels of metals in areas adjacent to tumors. Inductively coupled plasma-mass spectrometry (ICP-MS) was used for quantification of Cd, Fe, Zn, and Se.

RESULTS. Patients with biochemical (PSA) recurrence of disease had 12% lower median iron (95 μg/g vs. 111 μg/g; $P = 0.04$) and 21% lower zinc (279 μg/g vs. 346 μg/g; $P = 0.04$) concentrations in the normal-appearing tissue immediately adjacent to cancer areas. Differences in cadmium (0.489 μg/g vs. 0.439 μg/g; 4% higher) and selenium (1.68 μg/g vs. 1.58 μg/g; 5% higher) levels were not statistically significant in recurrence cases, when compared to non-recurrences ($P = 0.40$ and 0.21, respectively).

CONCLUSIONS. There is an association between low zinc and low iron prostate tissue levels and biochemical recurrence in prostate cancer. Whether these novel findings are a cause or effect of more aggressive tumors, or whether low zinc and iron prostatic levels raise implications for therapy, remains to be investigated. *Prostate 71: 1231–1238, 2011.* © 2011 Wiley-Liss, Inc.

KEY WORDS: **PSA recurrence; zinc; iron; selenium; cadmium**

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INTRODUCTION

With variable degrees of success and frequently contrasting conclusions, several epidemiologic, clinical, and experimental studies have tried to link heavy metal exposure to prostate cancer incidence. In contrast, to the best of our knowledge, there are no publications on the possible influence of heavy metals on disease progression and outcome once prostate cancer is established. Direct exposure of bronchial epithelium to cadmium-rich dust is recognized as a mechanism for lung carcinogenesis [1,2], but it is unlikely that cadmium reaches such high concentrations in the prostate. Cadmium overburden could theoretically cause more aggressive disease and studies have repeatedly shown increased cadmium concentrations in prostate of individuals with clinically manifested prostate cancer [3–5]. Even though Feustel et al. found an association between tumor grade and stage and elevated prostate cadmium burden more than 20 years ago [3,6], there are no reports in the literature that correlate prostate tumor behavior with cadmium overburden.

Iron overload in hemochromatosis is associated with higher risk of hepatocellular carcinoma [7]. Epidemiologic studies have also shown high serum ferritin saturation associated with higher overall cancer mortality [8,9]. High iron intake may be associated with prostate cancer [10] and colon cancer [11,12]. Iron is necessary for tumor growth in experimental animals [13]. In contrast, attempts to partially deplete iron with chelating agents have been inconclusive because chelating agents have anti-tumor effects on cancer cells even in the absence of iron overload [14].

Prostate cancer has been consistently associated with low prostate zinc [3–5,15,16] and there is sufficient information from in vitro experiments to suggest that low zinc would facilitate tumor growth [17] and cancer cell invasiveness [18,19]; however, to our knowledge, there is no clinical study addressing association between disease aggressiveness and zinc levels.

Low pre-diagnostic selenium levels in serum also seems to predispose to a higher incidence of prostate cancer [20], but again, there is no evidence that selenium levels influence the outcome after the disease is already established.

Other heavy metals besides iron, zinc, cadmium, and selenium have also been discussed in the prostate cancer literature, but the above are the four that have received most attention.

Knowledge about the role of environmental agents on cancer progression and metastasis is more important in prostate cancer when compared to most other cancers because cancer in this location is extremely common, but only a few men ever develop an aggressive disease. In the United States of America, even though 45–65% of 70-year-old men who die of other causes harbor microscopic foci of prostate cancer on autopsy, there is only a 20% lifetime probability that a clinically apparent tumor will occur [21,22]. With such a disproportion, it becomes at least as important to study environmental agents that are associated with progression and metastasis of disease as it is to study agents that cause its initiation and promotion.

Recently, we demonstrated that inductively coupled plasma-mass spectrometry (ICP-MS) is the method of choice for analysis of selenium, iron, cadmium, and zinc in formalin fixed paraffin embedded (FFPE) prostate tissue [23]. The technique allows simultaneous determination of these elements in a single experiment with high sensitivity and a wide concentration range. In that work, the determined levels of metals in FFPE tissue were compared with those in matching fresh tissue thereby allowing an estimate of the relative loss of each element in FFPE tissue processing. We found that these four metal ions withstand tissue processing by variable degrees, ranging from excellent for Se to progressively less for Zn. In the present work, the ICP-MS method for elemental quantitation was applied to compare concentrations of metals in the prostate tissues of patients who had favorable or adverse long-term outcome after radical prostatectomy. We did not sample areas of tumor but areas adjacent to tumor because the latter is more informative of the tumor environment than the cancer cells themselves, as cancer cells may lose the mechanism to accumulate or eliminate metals as differentiation declines.

MATERIALS AND METHODS

Subjects

The source of tissues was the Cooperative Prostate Cancer Tissue Resource (CPCTR; http://www.cpctr.info/index.html). We obtained formalin fixed paraffin embedded tissue blocks of prostatectomy samples of 40 patients with PSA recurrence, matched 1:1 (for race, age, Gleason grading score, and pathology TNM classification [24]) with tissue blocks from 40 patients without recurrence ($n = 80$; nested case–control study). All non-recurrence patients had more than 5-years post-prostatectomy follow-up. In order to investigate metal ion concentrations in the prostate microenvironment, rather than inside the tumor itself, levels of heavy metals were compared for biochemical recurrence and non-recurrence cases in histologically normal areas immediately adjacent to tumors from the same paraffin blocks, where the larger focus of tumor was found. For cutting paraffin blocks, a titanium knife was used in order to avoid contamination with the metals of interest. At least 5 mm distance from the tumor was kept for

cutting the tumor-free sample. Usually greater distance was allowed. To delineate the tumor area, a stained slide from the same archival paraffin block was used. For each individual pair of recurrence and non-recurrence, the Gleason grades, scores and histologic features were the same. In order to avoid bias, the researchers did not know which tissues belonged to the biochemical recurrence or non-recurrence cases until the metal measurements were completed. Further, blinding was introduced during the procedure of cutting the paraffin blocks, as the pathologist did not know the recurrence status of the cases. Serum samples from these subjects were not available for comparison. Studies with human tissues were approved and conducted in accordance with the policies of the Institutional Review Boards of the University of Illinois at Chicago and the Armed Forces Institute of Pathology (Washington, DC).

Materials

Ultra pure water (distilled deionized, 18 MΩ) was obtained using Milli-Q system (Millipore Corporation, Billerica, MA) and used for all preparations. Nitric and acetic acids of ''Optima'' grade were purchased from Fisher Scientific (Pittsburgh, PA). Stock solutions of Fe, Zn, Se, Cd, and Y were purchased from SPEX CertiPrep, Inc. (Metuchen, NJ). Enriched selenium isotope (^{74}Se) was purchased from Isoflex (San Francisco, CA). Standard reference material (SRM), Bovine Liver-1577b was purchased from National Institute of Standards and Technology (Gaithersburg, MD).

Tissue Samples Preparation

Tissues were processed and analyzed as previously described [23]. In summary, tissue specimens were originally subjected to standard 10% buffered formalin fixation and paraffin embedding in a tissue processor. Tissue portions from the blocks were cut out with a titanium knife and deparaffinized with xylene at 55°C for 1 hr in a tissue processor. Upon deparaffinization, the tissue samples were dried in a vacuum chamber at 50°C overnight. Each dried sample was divided into three portions (5–10 μg) for triplicate analysis.

For ICP-MS analysis, each tissue portion for standard reference material (SRM), Bovine Liver-1577b; National Institute of Standards and Technology (Gaithersburg, MD) of similar weight for quality control was mixed with 20 μl of 1 mg/L Y, 20 μl of 5 mg/L ^{74}Se, serving as internal standards, and 0.3 mL of 70% nitric acid in Teflon 3-mL Micro Vessels D (CEM Corp., Matthews, NC). The samples were incubated at room temperature overnight followed by digestions at 110°C for 30 min in MARS-X microwave accelerated decomposition system (CEM Corp.). These digested tissue solutions were diluted with water and acetic acid to adjust the concentrations of internal standards to 10 μg/L for Y and 50 μg/L for ^{74}Se and 3% for acetic acid.

ICP-MS Analysis of the Samples

The tissue samples were analyzed using Thermo Finnigan Element2 ICP-mass-spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with a micro-concentric nebulizer (Micromist, Glass Expansion, Inc., Pocasset, MA) and a cyclonic spray chamber (Tracey, Glass Expansion, Inc, Pocasset, MA). For quantification, we used standard solutions containing 0–500 μg/L of Fe and Zn, 0–25 μg/L of Se, and 0–1.25 μg/L of Cd; and the same composition of the solvents as that in the digested samples. High-resolution setting of 10,000 or more was used in order to separate the $ArAr^+$ interference at m/z 76 for ^{76}Se, and the ArCl inference at m/z 77 for ^{77}Se. All elemental concentrations are calculated and presented on a dry prostate weight basis. Additional technical details, optimal plasma operating and mass-spectrometer acquisition parameters are listed in previous publication [23]. Each sample was analyzed as a triplicate of the sub-samples, in two acquisition runs per the solution, averaging the obtained concentration values.

Statistical Methods

The confounding factors of tumor grade, pathology TNM classification, race and age were controlled for by matching the recurrence and non-recurrence samples.

The association of each individual metal with prostate cancer recurrence was evaluated by the Wilcoxon signed rank test using paired differences of each recurrence case with its matched non-recurrence control case. This test provides a comparison of median metal concentration between the two groups. Bootstrapping was then used to compare the 25th and 75th percentiles of metal concentration between recurrence and non-recurrence groups. This set of comparisons is based on estimated difference in percentiles and associated bootstrapped 95% confidence intervals. Logistic regression was used to determine the joint association of the heavy metals with cancer recurrence. The Generalized Estimating Equations (GEE) model was used to account for the correlated nature of the data. All analyses were performed using SAS (version 9.1, SAS Institute, Cary, NC) and S-Plus 6.1. P values were considered significant if <0.05.

The finding of a trend for a worse outcome in individuals with cadmium overburden prompted a more focused examination of this metal by running a bootstrap analysis. The original data were re-sampled 1,000 times in S-Plus (MathSoft, Seattle, WA) in order to look

for differences between recurrence and non-recurrence groups at 25th and 75th percentiles (equivalently, the 1st and 3rd quartiles).

A logistic regression model was applied to the entire data set in order to address the possibility that prognosis could be affected by interaction among the concentrations of the various metals. We used this logistic regression model to address the possibility that zinc and cadmium work in opposition since these two elements, and possibly selenium, compete for metallothionein and other common carriers inside the cell [25].

RESULTS

Table I summarizes the levels of metal ions in the tissue samples. Figure 1 shows the differences in concentration of the metal ions in each of the 40 individual pairs of recurrence/non-recurrence tissue samples. For each metal there is a wide range of variation from pair to pair. The diagonal lines in Figure 1 indicate where the points would fall if the concentrations of each metal were identical in recurrence and non-recurrence tissue samples. For iron and zinc, lower concentrations were associated with recurrence (i.e., more points below the diagonal line). These differences in zinc and iron were not large, but they were statistically significant. Differences are more meaningful when one considers that these 40 pairs were already pre-matched for important variables that could have influenced results: tumor grade, pathology TNM classification, race and age. The graphs in Figure 1 also show a wide inter-individual variation in concentration for each one of the metals, part due to biological reasons and part due to pre-analytical variations in tissue fixation and processing. Despite these variations, statistical significance was detected for iron and zinc levels. For selenium, the expected protective effect was not apparent and surprisingly there was a trend for worse prognosis when selenium was elevated; however, these differences in selenium were not statistically significant.

Individuals with cadmium overburden showed a trend for a worse outcome but this difference was also not statistically significant. Further bootstrap analysis confirmed that there was no significant difference between the two groups at the 1st quartile (estimated difference at the 25% percentile was 31.9; 95% confidence intervals = −55, 152.5). However, at the 3rd quartile, significant differences were found (estimated difference at the 75% was 195.7; confidence intervals = 20.8, 509.5), where cadmium in the recurrence group is greater than cadmium in the non-recurrence group.

Logistic regression modeling found no statistically significant interactions between the metal ions.

Using the same statistical method (Wilcoxon paired rank test) that linked low iron or zinc concentrations as factors for higher risk for recurrence in this population, three markers of recurrence fared worse than the prostate level of these two metals: pre-operative serum PSA values ($P = 0.16$), tumor largest diameter ($P = 0.32$), and perineural invasion ($P = 0.50$). Positive margin of resection was a significant predictor of recurrence ($P = 0.02$).

DISCUSSION

Our study explored the association between levels of metal ions, in normal appearing areas of the prostate adjacent to prostate cancer, and biochemical recurrence of the disease after prostatectomy. While studies have been performed to investigate the relationship between metal ions and prostate cancer incidence, to the best of our knowledge, this is the first to show decreased iron and decreased zinc as markers of increased risk for PSA recurrence. The association of low iron and low zinc with adverse outcome was found in spite of the variability inherent to measurement of metal ion concentrations in FFPE tissues and wide variability in the concentrations of these metals.

Because the study design was a single-observation point, it does not answer the question of which

TABLE I. Prostate Cancer Outcome and Tissue Levels of Metal Ions

	Recurrence cases, median (μg/g)	Non-recurrence cases, median (μg/g)	% Difference between pairs, median[a]	*P*-value*
Iron	95	111	−12	0.04
Zinc	279	346	−21	0.04
Cadmium	0.488	0.439	4	0.40
Selenium	1.68	1.58	5	0.21

[a]Median/of/the % difference calculated from 40 pairs $= \frac{(\text{recurrence-non-recurrence}) \times 100}{\text{non-recurrence}}$.

*P-value obtained by the Wilcoxon signed rank paired test was significant for iron and zinc.

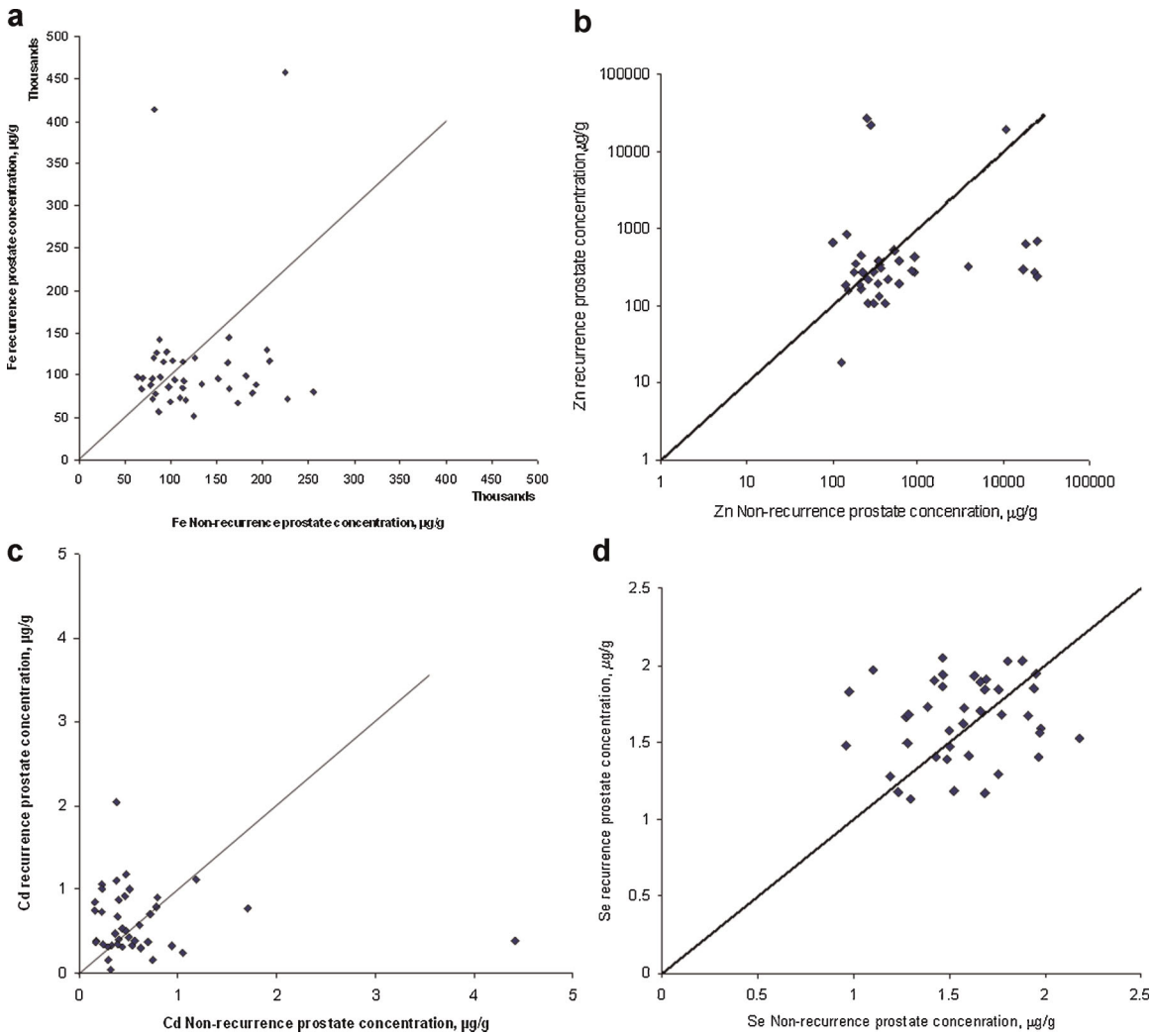

Fig. 1. The *Y*-axis shows the heavy metal values of recurrence member of each pair, the X-axis shows the values of corresponding matched non-recurrence member. There are 40 data points in each plot. The diagonal line has values $x = y$. If there are more data points below the line, the value is greater in non-recurrence group than it in recurrence group. Log transformed values were used for the zinc concentration graph. **a**: Comparison of iron levels in recurrence and non-recurrence prostate tissue. **b**: Comparison of zinc levels in recurrence and non-recurrence prostate tissue. **c**: Comparison of cadmium levels in recurrence and non-recurrence prostate tissue. **d**: Comparison of selenium levels in recurrence and non-recurrence prostate tissue.

abnormality came first; the changes in metal concentration or tumor aggressiveness. This distinction is important from the point of view of clinical applicability such as chemoprevention; because low iron, low zinc, and high cadmium are amenable to treatment. If instead, these changes are a consequence of more aggressive tumors, these findings could potentially be used to help therapy decision making, such as prescribing additional, adjuvant therapy to individuals with higher risk of recurrence. Measures of selenium tissue levels showed interesting and perhaps unexpected negative results in outcome prediction, therefore requires further investigation.

The normal prostate has the highest zinc concentration of any organ [26]. Prostates that harbor cancer have several-fold lower zinc, as consistently shown in previous studies [3–5]. Low serum zinc level in prostate cancer has even been proposed as an adjunct to serum PSA for the diagnosis of prostate cancer [20]. Our study adds to the evidence around the relationship between prostate cancer and zinc by finding a difference in outcome in cancer patients depending on prostate zinc concentration.

In physiologic concentrations in vitro zinc exposure causes a pro-apoptotic effect on prostate cancer cells [16], a possible explanation of why low zinc would favor a bad clinical outcome. Zinc also decreases prostate cancer cell in vitro invasion [19]. Several of the therapeutic targets that have been clinically linked to prostate cancer recurrence, such as NF-κB, VEGF, IL-6,

IL-8, and MMP-9 have reduced expression in cells treated with physiologically relevant concentrations of zinc [27]. The in vivo effect of low zinc on the behavior of prostate cancer cells in experimental models has not been determined. Our results suggest that low zinc would favor biochemical recurrence, but we cannot rule out the possibility that low zinc is a consequence of more aggressive tumors rather than the cause. Zinc transport in prostatic epithelium is a very specific function of normal, terminally differentiated cells, and it is not surprising that less differentiated tumors would show lower zinc levels. It should be noted, however, that zinc concentration was measured not in the cancer cells, but in areas adjacent to the cancer, therefore loss of differentiation would not explain low zinc content. Moreover, by the experimental design of nested case–control study, the tumors in each pair had the same histologic Gleason grade score.

Based on our previous study that zinc transporters and tissue zinc are lower in African Americans than in European Americans [28], we designed the study to compare only individuals of the same race against each other. It is a controversial issue whether or not patients of any race should take zinc supplements. Zinc overload can cause toxic effects in humans, such as renal and neurological damage [29]. Moreover, a study involving 46,000 health professionals [30] found that men who consumed more than 100 mg/day of supplemental zinc had a higher (2.9-fold) rather than lower relative risk of advanced prostate cancer. Clearly, there is gap in knowledge regarding the effect of zinc supplements in patients with prostate cancer.

Lower tissue iron in the areas adjacent to tumors with a worse outcome is unexpected because much of the literature on iron and prostate cancer is directed at the negative effect of excess iron, and the possibility that excess iron causes oxidative stress [31]. Iron overburden is clearly associated with hepatocellular carcinoma as illustrated in patients with hemochromatosis [7] and there are several epidemiological and clinical studies to suggest that low iron may decrease the incidence of cancer in general and also of prostate cancer [8,9,32]. In an apparent contradiction to these studies, Kuvibidila reported evidence to suggest that prostate cancer is not associated with increased body-iron stores [33], an interesting finding that opens more questions than it answers [34]. One possibility is that high iron predisposes to prostate cancer, but once cancer is established, it would induce a decrease in body-iron stores, and/or a decrease in metabolically available iron.

Regulation of total body iron metabolism is very complex. There are various metabolic checkpoints to avoid iron overburden and only minimal amounts of iron can be physiologically eliminated. One of the possible mechanisms to explain our findings of low iron in individuals with worse prognosis is the interleukin-6 (IL-6) and hepcidin axis that regulates iron metabolism. Prostate cancer patients with worse prognosis have more IL-6 [35,36] in the circulation. This cytokine is known to induce the production of hepcidin in the liver [37]. One of the main roles of hepcidin is to decrease iron export from intestinal epithelial cells and iron-storing macrophages into the circulation, effectively decreasing total available iron for hematopoiesis [38]. This explanation, although plausible, needs further testing. An alternative explanation is that low iron level in the prostate microenvironment, present even before prostate cancer starts, would cause a more aggressive outcome of the disease. This latter hypothesis would hold important implications for therapy and chemoprevention.

A 4% cadmium excess found in our cases with biochemical recurrence was not statistically significant, although a bootstrap analysis showed that the difference was significant at the 75th percentile. This discrepancy could be due to the fact that the Wilcoxon signed rank test compares the median (50th percentile) metal concentration. These results show the need for additional studies to clarify the possibility of an association. Unlike iron, zinc and selenium, cadmium is not a necessary nutrient. This environmental agent is elevated in the blood and prostate tissue in men with prostate cancer [3–5]. In addition, cadmium has a well-recognized role in human lung cancer and in extremely high doses it act as carcinogen in experimental animals [1]. Cadmium has long been suspected as a risk factor for prostate cancer although its concentrations in prostate clinical samples are well below the concentrations that have been shown to be carcinogenic in experimental in vivo prostate cancer models [39].

While the question of whether cadmium can cause prostate cancer remains unresolved, there is some evidence that cadmium increases the aggressiveness of cancer in experimental models [40–42], and possibly in humans [43]. Therefore, it could be expected that prostatic cadmium overburden may contribute to a worse disease outcome.

In contrast to cadmium, selenium is an essential trace element and a necessary component of a large number of proteins. In vitro work has established that non-protein selenium metabolites, typically at micromolar concentrations, can differentially suppress prostate tumor cell growth, induce apoptosis, and inhibit proliferation-associated signaling pathways [44,45]. We did not find any association between selenium and outcome, despite anticipating that elevated selenium would have protected against cancer progression. However, this lack of association could be explained by our measurement of total selenium ion

concentration; as opposed to a selenium speciation analysis. In the body selenium is present in various forms such as catalytic selenite, selenocystamine, and selenomethionine all of which have different biological functions [46]. Selenium speciation should occur in future studies.

The methodological limitations of this study need to be taken into consideration. In part due to the retrospective nature of the study, we did not have serum samples collected, diet questionnaires, or occupational health data for a more comprehensive study. The results for cadmium and selenium could be a consequence of the wide inter-individual variation in the level of these metals and therefore a larger cohort could show statistical differences between subjects with PSA recurrence and non-recurrence, especially for cadmium levels, where bootstrap analysis showed the possibility of higher values predicting a bad outcome. Finally, even though we have previously shown that paraffin blocks could provide metal levels that correlate well with the levels in the fresh prostate, the correlation was not perfect [23].

CONCLUSIONS

The authors believe that these findings should initiate further research into a largely unexplored area; the effect of environmental agents on prostate cancer progression. In terms of modifiable risk factors, there is mounting evidence that a diet rich in animal fat may accelerate prostate cancer progression [47]. Adding to this field, data presented here suggest that metal ions or deficiency of metal ions may also influence cancer outcome. For prostate cancer, the study of agents that cause tumor progression and metastasis may be even more important than for other types of cancer. Further study is particularly warranted because, as cited above, a large percentage of otherwise normal older individuals have clinically inconsequential but already established prostate cancer, but only a few will develop aggressive disease. The data presented here suggests that low iron, low zinc and possibly high cadmium concentrations may have the ability to turn these ''benevolent'' tumors into more aggressive tumors. Alternatively, especially in the case of iron, these changes in metal levels may simply be secondary markers of more aggressive tumors. Further research in this area will help explain the significance of these findings.

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